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THE EFFECT OF PROLACTIN ON S^{35} FIXATION IN THE COSTAL CARTILAGE OF THE HYPOPHYSECTOMIZED RAT

CHARLES W. DENKO[1]

Department of Medicine and the Argonne Cancer Research Hospital, United States Atomic Energy Commission, University of Chicago, Chicago 37, Illinois

ABSTRACT

Prolactin and growth hormone were studied in their effect on the binding of radiosulfur, S^{35}, in the costal cartilage of the hypophysectomized rat. Both hormones produced a significant increase in the S^{35} fixation of the costal cartilage of hypophysectomized male and female rats. Gradually increasing binding of S^{35} occurred with the increasing doses of prolactin. The normal male rat exhibited no change in S^{35} uptake when treated with prolactin. The increase in S^{35} fixation occurring in prolactin-treated rats was much smaller than the increment in S^{35} incorporation observed in animals receiving growth hormone. When both hormones were administered together no alteration in the S^{35} fixing pattern was noted.

STUDIES of the effect of gonadotrophins on the skeletal system indicate that prolactin or lactogenic hormone increases the growth of the skeleton (1), (2). Geschwind and Li (3) reported that the stimulatory effect of prolactin on the tibial cartilage is sex-dependent, occurring only in the hypophysectomized male and not in the hypophysectomized female rat. They also found that the maximal response of the tibial cartilage to prolactin was lower than that obtained with growth hormone.

In an investigation of the growth hormone effect on S^{35} fixation in the cartilage of hypophysectomized rats (4) the question of prolactin contamination arose. Since the currently used methods for preparing each of the hormones are such that contamination is highly unlikely (5) an investigation was undertaken to determine the effect of prolactin itself on S^{35} uptake by the costal cartilage of the hypophysectomized rat.

The sex-dependency of the response to prolactin was studied by measur-

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[1] Present address: Rackham Arthritis Research Unit, The University of Michigan Hospital, Ann Arbor, Michigan.

ing S^{35} incorporation by costal cartilage in hypophysectomized male and female rats. Normal rats were given prolactin to determine its effect on S^{35} binding in their costal cartilage. To compare the influence of growth hormone and prolactin in hypophysectomized rats the hormones were given to similar groups of animals and the S^{35} bound in the costal cartilage was determined.

METHODS

The rats used were obtained from Hormone Assays, Inc., Chicago, Illinois. Both male and female animals were hypophysectomized at approximately age 22 days, then were observed for fourteen days to evaluate completeness of hypophysectomy. Those that continued to gain weight at a rate appreciably higher than the other rats in the group were not used. Ordinarily the average weight gain did not exceed five grams. Female rats weighed about 55 to 60 gm. when they were used experimentally while the male rats were 5 to 10 gm. heavier. Further observations of completeness of hypophysectomy were made at autopsy by examination of the target endocrine organs which remained small.

The normal male rats were litter mate controls. All animals were kept in an air-conditioned laboratory under similar conditions of housing and feeding. The hypophysectomized animals received a mixture of canned dog food, evaporated milk and bread mixed thoroughly in a mechanical blender. Tap water and laboratory pellets were provided *ad libitum*. In addition a supplement of lettuce and carrots was given daily. The animals were observed daily and those that developed upper respiratory infections were removed from the study. Only the normal and the growth hormone-treated rats demonstrated significant weight gains.

All animals received 10 microcuries of S^{35} in 0.2 ml. of saline intraperitoneally at the time that the hormone was given. Hormone solutions were administered in 0.5 to 1.0 ml. of saline intraperitoneally except for the fifteen units of prolactin which was injected in 2 ml. Control animals received saline equal in volume to the hormone solution. All preparations were given daily for four consecutive days with the animals being sacrificed by etherization 24 hours after the last dose. The cartilage was dissected clean and radioactivity determined to a probable error of 3 to 5% in a windowless gas-flow counter in a manner previously described (4). In the experiments with the hypophysectomized rats three groups of four or five animals were used at each dose level.

The radiosulfur was obtained carrier-free as inorganic sulfate from the AEC National Laboratories, Oak Ridge, Tennessee. Batch levels contained radioactivity $\pm 20\%$ of the stated amount. No attempt was made to standardize the different batches. Correction was made for radio-decay. The hormone preparations were obtained from Armour Laboratories, Chicago, Illinois, as follows: growth hormone lots R377243 and R491132 containing negligible amounts of thyrotrophin and pressor substances as impurities; prolactin lots 491153 and 759–39BB containing 20–25 I. U. per mg. with ACTH of <0.05 units/mg. The latter prolactin preparation did not produce a significant gain in body weight when 150 μg. was administered to a hypophysectomized female rat (5).

RESULTS

Prolactin, when administered intraperitoneally, produced a significant increment in the amount of S^{35} localized in the costal cartilage of the hypophysectomized female rat (Table 1). This stimulation of S^{35} uptake increased with increasing doses of the hormone. No appreciable influence on S^{35} incorporation was detectable with the smallest dose of prolactin,

TABLE 1. EFFECT OF PROLACTIN ON S^{35} FIXATION IN THE COSTAL CARTILAGE OF THE HYPOPHYSECTOMIZED FEMALE RAT

	Control	Prolactin			Growth hormone 17 μg.
	Saline	1/13 unit	1 unit	5 units	
No. of Animals	14	9	11	7	8
Mean* ± S. D.	870 ± 180	950 ± 120	1310 ± 590	1820 ± 470	2180 ± 510
Range*	610–1270	740–1080	670–3000	1010–2580	1490–3190
Significance of comparison with control		P < 0.2	P < 0.02	P < 0.01	P < 0.01

* In S^{35} counts per min. per 100 mg. of wet tissue.

one-thirteenth unit, 3.4 μg. However, a response was apparent when one unit, 45 μg., was administered. An S^{35} level appreciably higher than the one unit level was attained when five units, 225 μg., of prolactin was tested. When only seventeen mcg. of growth hormone alone was injected the resultant stimulation of S^{35} uptake over that of the controls was approximately equivalent to that produced by the five units or 225 μg. of prolactin. Thus on a weight for weight basis the stimulation of S^{35} fixation induced by prolactin is only 8% of the growth hormone effect. That the S^{35} binding capacity of cartilage is not the limiting factor in response to prolactin is demonstrated by the further response of cartilage to 100 μg. of growth hormone.

Prolactin produced a significant elevation in the S^{25} levels of the costal cartilage of the hypophysectomized male rat (Table 2). As in the female rat the increment in S^{35} uptake increased with increasing dosage of prolactin. At the one unit level the augmented response over mean control values was essentially 50% in both male and female rats. At five units similar rises over the mean control levels again were noted, 105% in the male and 110% in the female. Thus the hypophysectomized female rat closely resembles the hypophysectomized male rat in the prolactin stimulation of S^{35} fixation in the costal cartilage.

TABLE 2. EFFECT OF PROLACTIN ON S^{35} FIXATION IN THE COSTAL CARTILAGE OF THE HYPOPHYSECTOMIZED MALE RAT, THE GROWTH HORMONE TREATED HYPOPHYSECTOMIZED MALE RAT, AND THE NORMAL MALE RAT

	Hypophysectomized						Normal Rat	
	Control	Prolactin (PL)			Growth Hormone (GH)		Control	Prolactin
Dose	NaCl	1 unit	5 units	15 units	100 μg.	100 μg. PL—7.4 U	NaCl	5 units
No. of animals	13	9	11	13	14	9	6	7
Mean* ± S.D.*	1930 ± 490	2900 ± 1240	3960 ± 1600	6200 ± 1700	9000 ± 2500	7360 ± 2940	3160 ± 610	3790 ± 1390
Range*	1030–3000	1420–3770	2200–7450	3300–8900	4500–13700	2600–11300	1820–3600	2500–6100
Significance of Comparison (a) with control		P < 0.05	P < 0.01	P < 0.01				P < 0.4
(b) with GH 100 μg.				P < 0.01		P < 0.2		

* S^{35} counts per min. per 100 mg. of wet tissue.

As much as 15 units of prolactin did not effectuate the maximal S^{35} binding in the costal cartilage since additional stimulation was attained during growth hormone treatment (Table 2). No definite prolactin influence was noted on the S^{35} incorporation promoted by growth hormone in rats receiving both hormones simultaneously. When the normal rat containing presumably normal levels of endogenous growth hormone was given five units of prolactin no significant alteration in the S^{35} levels of the cartilage was observed.

DISCUSSION

In some respects these results are at variance with previously reported studies on prolactin or lactogenic hormone. Some explanation may be found in the differences in the preparations of hormone studied or in the sensitivity or specificity of the indicator systems used. The lack of definite summation of effects on S^{35} binding when growth hormone and prolactin were administered together may be due to the wide spread in animal responses noted here. No attempt was made to evaluate the effect of prolactin on specific organs in the male rat since no gross changes were found at autopsy. Previous investigators reported no detectable influence of lactogenic hormone upon organ weights, nitrogen and phosphorus balance, fat and protein content of liver and carcass in the male rat (6).

S^{35} incorporation into cartilage, a vital process reflects a synthesis of radioactive sulfomucopolysaccharides of the chondroitin sulfate type in the ground substance (7, 8, 9, 10). The stimulation of S^{35} incorporation into cartilage affected by growth hormone is also accompanied by a generalized increase in the body weight of the hypophysectomized rat (4). In this respect prolactin differs from growth hormone since the increase in S^{35} uptake in cartilage in prolactin-treated animals is not paralleled by an increase in the body weight of the hypophysectomized rat. Prolactin may have a nonspecific action in increasing the synthesis of sulfated mucopolysaccharides in connective tissue such as the cartilage, similar to its stimulating influence in other tissues.

Stimulatory actions of prolactin have been reported in the following increased parameters of growth of the pigeon crop sac: the pentose nucleic acid and succinic dehydrogenase (11), P^{31} and P^{32} fixation (12), number of epithelial cells and diffuse cytoplasmic basophilia (13). Another stimulatory action of prolactin has been observed in fish, in the hypophysectomized Fundulus, namely, the increased production of melanin (14). The diabetogenic action of prolactin (15) and its effect in increasing S^{35} fixation are similar to the action of growth hormone which produces both reactions much more potently. Perhaps the same metabolic pathways are affected in different degrees by these hormones. Perhaps the same target organs may be affected by different pathways. More knowledge of the molecular

structures of the active hormonal principles involved and mechanisms of S^{35} fixation are desirable.

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DEPLETION OF ADRENAL ASCORBIC ACID AND CHOLESTEROL: A COMPARATIVE STUDY[1]

R. L. ELTON[2], I. G. ZARROW AND M. X. ZARROW
Department of Biological Sciences, Purdue University, Lafayette, Indiana

ABSTRACT

The effects of ACTH and exposure to cold on the concentration of adrenal ascorbic acid and cholesterol were studied in several species. Exposure to severe cold produced significant depression of adrenal ascorbic acid in opossums and dogs, but failed to do so in frogs, toads, chickens, mice, hamsters, rabbits, and cats. ACTH failed to produce such a depletion in frogs, toads, chickens, rabbits, cats, or dogs; significant depressions were observed in opossums, mice and hamsters. Adrenal cholesterol concentrations remained unchanged in most species tested, however, increases were observed in rabbits, following cold exposure, and in frogs, following ACTH treatment.

THE need for additional data of a comparative nature is becoming increasingly more important in endocrinology. The value of information obtained from lower vertebrates and invertebrates bears out the importance of these animals both as objects of research and as a source of basic physiological information.

The role of ascorbic acid in adrenal physiology remains obscure. Early studies in rats showing adrenal ascorbic acid depletion following exposure to various stressors suggested that this vitamin was intimately related to the physiologic activity of the adrenal cortex (1, 2, 3). More recent findings have questioned the validity of this hypothesis (4, 5, 6, 7). Most studies designed to resolve this perplexing problem have employed laboratory rats or guinea pigs, largely ignoring other species.

Jailer and Boas (8) failed to demonstrate depletion of adrenal ascorbic acid in chicks treated with adrenocorticotropin (ACTH) or epinephrine. Similar findings were reported for the duck (9) and the quail (10). As these responses differ from those of rats additional comparative data seemed needed. Our experiments were designed to determine the effect of exogenous ACTH or exposure to cold upon adrenal ascorbic acid and cholesterol in a series of species.

EXPERIMENTAL

Ascorbic acid and cholesterol concentrations were determined according to the methods of Roe and Kuether (11) and Schoenheimer and Sperry (12) respectively.

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[2] Predoctoral Public Health Fellow, 1956–1957. Present address: Division of Biological Research G. D. Searle & Co., Chicago 80, Illinois.

Total and free cholesterol was determined and the esterified form was calculated as the difference between the total and free cholesterol.

Animals used in this study were: frogs (*Rana* sp.); toads (*Bufo*, sp.); fowl (*Gallus domesticus*); opossums (*Didelphis virginiana*); hamsters (*Cricetus auratus*); mice (Rockland all purpose Swiss); rabbits (grey chinchilla); cats and dogs. All animals were sexually mature males or females. In certain species where adequate numbers of one sex were not available the data from both males and females were combined as indicated in the tables. Wild animals were maintained in air-conditioned animal quarters for two to four weeks prior to use. Those animals to be used as controls were kept quietly without handling until the time of sacrifice thus reducing to a minimum any stimulation which might result in endogenous secretion of ACTH.

Animals were sacrificed one to four hours after receiving an intramuscular injection of ACTH (ACTHAR[3]) or following exposure to cold for one to four hours (0–5°C). The large animals were killed with a lethal dose of sodium barbital given intraperitoneally; the smaller animals were killed by decapitation. The adrenals were removed, usually within 2–5 minutes following death, quickly freed of extraneous tissue, weighed on a torsion balance and homogenized in grinding tubes containing suitable solvents for the subsequent determination of ascorbic acid or cholesterol.

RESULTS

In opossums and dogs ascorbic acid was depleted after cold exposure ($P < .05$ and $< .01$, respectively). ACTH produced significant decreases in adrenal ascorbic acid in three of the nine species tested (opossum, mouse, and hamster; $P < 0.01$). The other species (frogs, toads, chickens, rabbits and cats) were not affected by either cold or ACTH (Table 1).

Contrary to the expected fall in adrenal cholesterol concentration following exposure to low environmental temperatures, a marked *increase* in the concentration of this substance was found in the adrenals of rabbits ($P < 0.01$). Cold was ineffective in frogs, toads, chickens, opossums, cats and dogs (Table 2). Except for frogs where significant increases were obtained ($P < 0.01$) ACTH was ineffective in producing changes in cholesterol in the other animals tested; i.e., toads, chickens, opossums, rabbits, cats and dogs (Table 2). While some increases were observed in these latter species, these changes were not significant.

DISCUSSION

That the adrenals of several species failed to show significant changes in ascorbic acid and cholesterol following ACTH treatment or exposure to cold temperatures is not unexpected. The results observed in the chicks (8), ducks (9) and quail (10) indicated that avian species respond to stressors differently from rats (1, 2, 3). In the present study only opossums, mice, hamsters and dogs were found to undergo ascorbic acid depletion following stress. In the amphibians tested (frogs and toads) neither ACTH nor cold were effective in producing ascorbic acid depletion. As both of these animals are poikilotherms the failure of cold to produce a depletion

[3] Obtained through the courtesy of Armour and Co., Chicago, Illinois.

TABLE 1. EFFECT OF ACTH OR COLD EXPOSURE UPON ADRENAL ASCORBIC ACID CONTENT IN VARIOUS SPECIES

Species	Sex	Treatment	No. of animals	Average adrenal ascorbic acid (mg.%)	% change
Frog	Both	Control	5	40	
		ACTH 20 I.U.	6	35	−12
		Cold 0–5° C	6	43	+ 8
Toad	Both	Control	10	23	
		ACTH 20 I.U.	10	19	−17
		Cold 0–5° C	8	21	− 9
Chicken	Males	Control	21	142	
		ACTH 20 I.U.	16	146	+ 3
		Cold 0–5° C	19	143	+ 1
Opossum	Both	Control	16	210	
		ACTH 20 I.U.	10	171	−19**
		Cold 0–5° C	12	192	− 9*
Mouse	Females	Control	20	258	
		ACTH 4 I.U.	16	206	−22**
		Cold 0–5° C	9	270	+ 5
Hamster	Males	Control	26	143	
		ACTH 20 I.U.	19	114	−20**
		Cold 0–5° C	25	139	− 3
Rabbit	Females	Control	9	237	
		ACTH 40 I.U.	8	199	−16
		Cold 0–5° C	6	240	+ 1
Cat	Both	Control	8	120	
		ACTH 40 I.U.	7	105	−13
		Cold 0–5° C	6	145	+21
Dog	Both	Control	7	171	
		ACTH 40 I.U.	7	152	−11
		Cold 0–5° C	6	135	−21**

* Significant difference ($P < 0.05$) using Wilcoxon Rank Sum test (24).
** Significant difference $P < 0.01$.

may be due to a generalized decrease of all metabolic processes; adrenal requirements therefore being minimized. Thus cold probably should not be considered a stressor in these two forms. Following exposure to cold, the concentration of adrenal ascorbic acid decreased in only two of the nine homeothermic forms (opossums and dogs). These data suggest either that most of these species do not exhibit an adrenalcortical response to severe cold or that adrenal ascorbic acid depletion is not a satisfactory index of cortical activity.

Although ACTH was effective in causing adrenal ascorbic acid changes in more species than was exposure to cold, it was without effect in the majority of the animals tested (Table 1). Whether the data obtained, following cold exposure, indicate anything more than phyletic specificity is difficult to ascertain. Partial explanation for the inability of ACTH to produce the expected drop in adrenal ascorbic acid may be due to the route of administration. Woods was able to evoke ascorbic acid depletion in the wild

Norwegian rat with ACTH only if it was administered in extremely large intramuscular doses or when it was administered by slow intravenous-drip method; severe cold and noise being ineffective (13, 14, 15). The high doses of ACTH utilized in the present study tend to minimize the importance of the route of administration. In addition, the absence of a response following extreme cold, where endogenous ACTH was presumably secreted, suggests the existence of factors other than mode of administration.

TABLE 2. EFFECT OF ACTH OR COLD EXPOSURE UPON THE ADRENAL CHOLESTEROL (ESTER) IN VARIOUS SPECIES

Species	Sex	Treatment	No. of animals	Average adrenal cholesterol (mg.%)	% change
Frog	Both	Control	11	1.13	
		ACTH 20 I.U.	9	2.50	+121*
		Cold 0–5° C	9	1.54	+ 31
Toad	Both	Control	8	1.44	
		ACTH 20 I.U.	10	1.14	− 8
		Cold 0–5° C	9	2.09	+ 45
Chicken	Males	Control	20	3.66	
		ACTH 20 I.U.	18	4.29	+ 17
		Cold 0–5° C	20	4.61	+ 23
Opossum	Both	Control	9	0.51	
		ACTH 40 I.U.	7	0.51	0
		Cold 0–5° C	10	0.59	+ 15
Rabbit	Females	Control	17	5.58	
		ACTH 40 I.U.	7	7.63	+ 37
		Cold 0–5° C	10	9.85	+ 76*
Cat	Both	Control	13	5.12	
		ACTH 40 I.U.	7	5.07	− 1
		Cold 0–5° C	6	6.15	+ 20
Dog	Both	Control	7	4.89	
		ACTH 40 I.U.	9	4.80	− 1
		Cold 0–5° C	5	5.77	+ 18

* Significant Difference ($P < 0.01$) using Wilcoxon Rank Sum Test (24).

Absence of adrenal cholesterol (esterified) depletion following the treatments used in these experiments exemplifies the need for continued work with additional species. The concentration of this substance, thought to be a precursor of the adrenal steroids (16), was found to increase in rabbits and frogs (Table 2), and in no case was there significant depression. This lack of cholesterol depletion has also been noted in the mouse following ACTH treatment and intense noise (17, 18).

There exist several possibilities to explain the lack of adrenal response observed in this study. Decreased sensitivity to environmental changes (or an increased pituitary threshold) may be the responsible factor in those animals failing to respond to the stressors with adrenal ascorbic acid or cholesterol depletion. Woods suggested this to be the case in the wild rat

as opposed to its domesticated counterpart (13). In the present study, while depletion of adrenal ascorbic acid was observed in mice and hamsters following massive doses of ACTH, exposure to cold was without effect. Whether decreased sensitivity exists at the hypothalamico-pituitary level or at the adrenal level can not be ascertained; the failure of large doses of ACTH to produce changes suggests decreased adrenal sensitivity, while the lack of response to severe cold suggests both adrenal and hypophyseal envolvement. Furthermore, even though changes in sensitivity may be evident when comparing wild and domesticated rats, decreased sensitivity alone is not a satisfactory explanation for such species as the chicken, rabbit or cat which fail to show the adrenal responses, even though domesticated.

A second suggested explanation is that set fourth by Kessler and Leathem (17) who attribute the lack of adrenal cholesterol depletion in mice following ACTH treatment to a rapid *repletion* of cholesterol stores, a process implied to be going on concurrently with depletion with a balancing effect. If this obtains in the present study, relative depletion of ascorbic acid will be evident only in those species wherein repletion is limited; i.e., the mouse, hamster, opossum and dog. In the other species this hypothesis would imply that repletion occurs at a rate equal to or greater than depletion. Alpert notes that in hamsters the return of adrenal weight to normal values following treatment with ACTH occurs more rapidly than in rats or guinea pigs (19). This implies a more rapid recovery of the adrenals of hamsters. In this study, ACTH produced a 20 per cent depletion while Alpert obtained 26 per cent depletion under the conditions of his studies, both well below the 50% depletion observed in rats, suggesting that the rate of depletion: repletion is specific for each species.

A consideration, not to be discounted, is the possibility that a phyletic specificity may exist with respect to the ACTH used. Such chemical specificity has been shown to exist with bovine and porcine somatotropins (20, 21). Limited evidence is available (22) indicating such specificity to exist in chickens. It was found that while bovine ACTH was ineffective in chickens, extracts of chicken pituitaries produced marked adrenal stimulation as measured by deposition of glycogen in the liver of young chicks. However, since bovine ACTH has been shown to be an effective and, presumably, specific stimulant of corticoid secretion in dogs (23), the importance of such specificity is minimized.

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THE EFFECT OF ALTERATIONS IN THYROID FUNCTION ON THE PITUITARY GROWTH HORMONE CONTENT AND ACIDOPHIL CYTOLOGY[1]

JOLANE SOLOMON AND ROY O. GREEP

Biological Research Laboratories, Harvard School of Dental Medicine, Boston 15, Massachusetts

ABSTRACT

The pituitaries of female rats subjected to various alterations in thyroid function were examined for changes in hypophyseal growth hormone content and acidophil morphology.

Thyroidectomy caused a significant decrease in the amount of growth hormone as well as a decrease in the acidophil granulation and population, whereas subsequent treatment with thyroxine allowed the pituitary structure and growth hormone content to return to normal. The pituitaries of unoperated rats fed a diet containing thyroid powder contained less growth hormone than those from normal controls, and the population and granulation of the pituitary acidophils were decreased.

The pituitary growth hormone content of thyroidectomized rats fed a diet containing 2,4-dinitrophenol was increased above thyroidectomy levels, but neither the pituitary cytology nor the hormonal level was restored to normal. The same diet fed to unoperated rats did not significantly alter the pituitary growth hormone levels and structure.

SINCE thyroid deficiency causes a depression of somatic and skeletal growth, accompanied by degranulation of the pituitary acidophils, whereas thyroid replacement therapy restores body growth and pituitary structure, it has been presumed that the thyroid influences the elaboration of hypophyseal growth hormone (1, 2, 3, 4). The present investigation was designed to measure changes in the pituitary growth hormone content and cytology of female rats subjected to various alterations in thyroid function.

MATERIALS AND METHODS

One hundred female rats, 30 days of age, were randomized into five groups of twenty animals each and treated as follows:

Group 1. Thyroidectomized at 33 days of age, and maintained on Purina laboratory chow *ad libitum.*

Group 2. Thyroidectomized at 33 days of age, and fed a diet of ground chow containing 0.2% of 2,4-dinitrophenol (DNP) from the 56th to the 75th day of life.

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Group 3. Unoperated rats fed a diet containing 0.7% desiccated thyroid powder from the 45th to the 66th day of age. The thyroid powder supplement was changed to 0.6% for the remainder of the experimental period (day 67 to day 75).

Group 4. Unoperated rats maintained on a diet containing 0.2% DNP from the 74th to the 75th day of age.

Group 5. Intact controls maintained on Purina laboratory chow *ad libitum.*

In a second series, the effect of thyroxine therapy was examined. Thirty-day-old female rats were randomized into the following groups:

Group 6. Nine thyroidectomized rats were maintained on laboratory chow *ad libitum.*

Group 7. Twenty-four thyroidectomized rats were treated with daily subcutaneous injections of 90% L 10% D crystalline thyroxine (Squibb). From the 42nd to the 75th day of age, each rat received 5 μg. thyroxine per 100 gm. body weight per day.

Group 8. Nineteen unoperated females served as controls.

All operated rats were injected with 200 μc. of I^{131} at 45 days of age to assure complete destruction of thyroid tissue; and, on the day before they were killed with ether, they were injected with 10 μc. of I^{131}. At necropsy, the tracheal region adjacent to the site of operation was removed, dissolved in 2N NaOH, plated on planchets, and counted for radioactivity to determine the amount of active residual thyroid tissue. All animals were killed on the 75th day of age. The ovaries, uterus, adrenals, pituitary and thyroid were excised and weighed on a Roller-Smith balance. Examination of the tracheal region of thyroidectomized rats revealed no residual thyroid activity.

To study changes in pituitary growth hormone potency and cytology, the whole pituitaries were either placed in absolute acetone, dried and stored in a vacuum desiccator for use in the tibia test assay for growth hormone, or fixed in Bouin's solution for histological preparation.

The growth hormone potency of the pituitaries was determined according to the procedure of Evans *et al.* (6), using the parallel line method. For each assay three groups of hypophysectomized rats received graded doses of a purified beef growth hormone preparation (Armour and Co., #732–181); the dried pituitary material from each experimental group was injected at two dose levels. The pituitary suspensions were prepared by triturating the whole dried pituitaries in saline. Twenty-four hours after the four-day injection period, the animals were killed with ether, and the right tibia removed from those rats in which gross inspection of the sella turcica and organ weights evidenced completeness of hypophysectomy.

The pituitaries which were prepared for histological examination were stained with Gomori's aldehyde fuchsin (7) after oxidation with acidified permanganate, and counterstained with Halmi's method (8), using twice the amount of light green SF yellowish, and adding the chromotrop 2R. Photomicrographs were taken in the central portion of the pars distalis abutting on the intermediate lobe. Especial attention was paid to changes in acidophil granulation and population which were so striking that cell counts were unnecessary.

RESULTS

When the statistically evaluated growth hormone potency of the pituitary suspensions was compared with the histological structure of the glands, it was found that the measure of growth hormone in the hypophysis was generally relatable to the morphological changes in the pituitary.

The average organ and body weights of the several donor groups are shown in Table 1. The response of the epiphyseal plate and statistical evaluation of the assayed growth hormone potency of the pituitaries are found in Table 2.

TABLE 1. AVERAGE ORGAN AND BODY WEIGHTS OF DONOR RATS

Group	Treatment	No. rats	Ovaries	Uterus	Adrenals	Thyroid	Pituitary	Body weight
			mg.	mg.	mg.	mg.	mg.	gm.
1	Thyroidectomy alone	11	45	257	25	—	9.4	126
2	Thyroidectomy + DNP diet	20	20	73	22	—	6.6	93
3	Unoperated + thyroid diet	11	53	104	67	9.5	6.5	157
4	Unoperated + DNP diet	17	25	50	32	9.0	5.0	125
5	Unoperated control	18	59	276	53	11.0	9.7	189
6	Thyroidectomized alone	9	54	279	41	—	10.1	169
7	Thyroidectomized + thyroxine	24	72	243	57	—	8.8	185
8	Unoperated control	19	63	316	51	10.4	8.7	202

The data in each assay reported here are portions of two larger assays, in which the residual degrees of freedom were 88 and 115 respectively. In each assay, a combined slope was calculated from the responses to the standard beef growth hormone and test pituitary extracts (9), and lambda, calculated from the combined slope $\left(\frac{\text{standard deviation}}{\text{combined slope}}\right)$ was 0.32 and 0.28 respectively. The estimate of growth hormone potency for each group of pituitary extracts (expressed in μg. of growth hormone per mg. pituitary)

TABLE 2.* GROWTH HORMONE ASSAY OF DONOR PITUITARIES

Group	Source of pituitary preparation	Dose pituitary powder μg./day	Recipients No. of hypox. rats	Epiphyseal width of recipients micrs mean ±S.E.	μg. Growth hormone per mg. pituitary: Estimate ± % S.E.	95% confidence limits
	Assay 1					
1	Thyroidectomized	250	9	205± 9	26.8±36.1%	13.8–92.0
	Thyroidectomized	500	5	223±12		
2	Thyroidectomized + DNP diet	250	7	228±10	65.6±29.8%	39.2–110.0
	Thyroidectomized + DNP diet	500	7	265±10		
3	Unoperated + thyroid powder	250	5	242±12	75.2±35.5%	41.5–138.4
	Unoperated + thyroid powder	500	4	262±14		
4	Unoperated + DNP diet	250	5	250±12	107.0±35.7%	71.6–201.8
	Unoperated + DNP diet	500	5	278±12		
5	Unoperated control	250	9	267± 9	170.0±36.2%	97.4–331.7
	Unoperated control	500	6	296±11		
	Beef growth hormone	10	8	218±10	combined slope =84.83	
	Beef growth hormone	25	8	254±10	slope of standard =71.9	
	Beef growth hormone	50	8	266±10	standard deviation =27.12	
					λ = 0.32	
	Hypophysectomized controls	—	9	175± 9		
	Assay 2					
6	Thyroidectomized	250	5	242±14	60.3±28.6%	36.4–98.7
	Thyroidectomized	500	5	287±14		
7	Thyroidectomized 5 μg. thyroxine	250	9	267±10	100.0±24.7%	66.8–160.4
	Thyroidectomized 5 μg. thyroxine	500	8	308±11		
8	Unoperated control	250	9	273±10	104.0±24.9%	67.0–175.3
	Unoperated control	500	8	309±11		
	Beef growth hormone	10	8	239±11	combined slope =109.10	
	Beef growth hormone	25	8	264±11	slope of standard = 88.7	
	Beef growth hormone	50	8	302±11	standard deviation = 30.54	
					λ = 0.28	
	Hypophysectomized controls	—	9	158±10		

* The authors would like to acknowledge the kind assistance of Dr. Mindel C. Sheps for her advice on the statistical methods.

and its 95% confindence limits (Table 2) were calculated according to the usual method (9). In each assay, the responses to each test substance, when analyzed for nonparallelism, failed to demonstrate a significant departure from parallelism.

The epiphyseal responses to the pituitary extracts from each group were compared by performing the "t" test on the differences between the overall means for each group (e.g. Table 2: Group 1 mean response (214 ± 7.24) less Group 2 mean response (247 ± 7.24) $= 33 \pm 10.25$ and "t" $= 3.22$, $P < .01$).

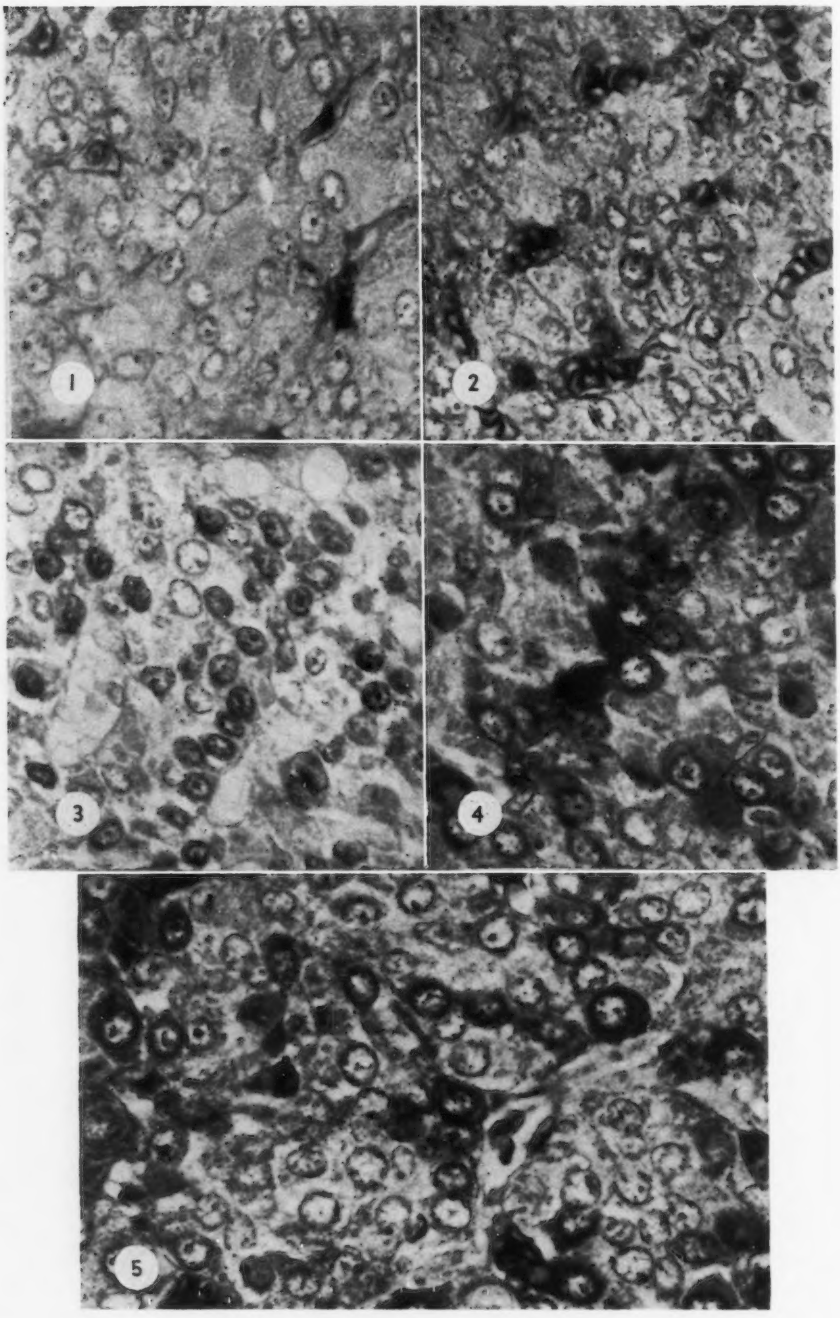
Thyroidectomy alone. These rats (Groups 1 and 6) failed to gain as much weight as their controls, and the growth hormone content of the pituitaries was depressed below normal levels ($P < 0.001$ and $P < 0.5$ respectively). Fig. 1 illustrates the decrease in acidophil population and granulation caused by thyroidectomy (cf. Fig. 5, normal control).

Thyroidectomy plus DNP in the diet for 18 days. This regimen was suggested by the finding of Goldberg *et al.* (5) that 0.2% DNP in the diet of PTU-treated rats for 30 days prevents the degranulation of pituitary acidophils. In the present experiment (Group 2), the body and organ weights were well below those of the controls. Although there was a significant increase in the growth hormone potency of these pituitaries when compared with the pituitaries of untreated thyroidectomized rats ($P < 0.01$), the total growth hormone content remained significantly lower than that present in the glands of unoperated controls ($P < 0.001$). Morphologically the pituitaries retained the characteristics which follow thyroidectomy (Fig. 2): the acidophils were degranulated, and the beta basophils were typically hypertrophied.

Unoperated plus thyroid powder in the diet for 30 days. On this treatment (Group 3), the body, gonad, uterine and pituitary weights were less than those of the controls; but the adrenal weights were greater. The growth hormone potency of the pituitaries was significantly less ($P < 0.01$) than those of the controls, and the acidophils (Fig. 3) were smaller and less granular than those of control pituitaries (Fig. 5). These changes in pituitary acidophil morphology of thyrotoxic rats were noted by Neuhaus (10) and confirmed most recently by Halmi (11).

Unoperated plus DNP in the diet of 30 days. The body weights of these animals (Group 4) were depressed below control values, but assay of the pituitaries obtained from this group demonstrated that the growth hormone content was not significantly different ($P > 0.1$) from that of the normal rats. Histological examination of these glands showed no noticeable change from the normal structure.

Thyroidectomy plus thyroxine for 33 days. These rats (Group 7) grew at a near normal rate, and the relative potency of the pituitaries was not significantly different from that of the controls ($P > 0.1$); however, it did not reach a significant increase over thyroidectomy levels ($P > 0.05$). Histo-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logically, the acidophils (Fig. 4) were normally granulated, and the population was not noticeably different from that of the controls.

DISCUSSION

Studies on the relationship of thyroid function to the growth rate, pituitary morphology and growth hormone content have shown that thyroidectomy results in a decrease in the growth rate (2), pituitary acidophil granulation (12), and growth hormone potency of the pituitary (13, 14), whereas thyroxine therapy restores the growth rate and pituitary cytology (2).

The present experiments demonstrate that the growth hormone potency as well as the pituitary structure may be restored to normal values in thyroidectomized rats treated with thyroxine therapy. These results suggest that the content of pituitary growth hormone is influenced either by the circulating level of thyroxine or by some metabolic action of the thyroid hormone. Further, the decrease in body growth, pituitary acidophil granulation and growth hormone content of hyperthyroid rats (Group 3) lends additional support to the hypothesis that pituitary growth hormone production is sensitive to thyroid function.

In these experiments the measured growth hormone content was significantly decreased below control values when the pituitary acidophils were degranulated (Assay 1: Groups 1, 2 and 3 each versus Group 5; Assay 2: Group 6 versus Group 8). However, on the basis of the cytological techniques employed, pituitary acidophil degranulation was not quantitatively related to the amount of assayable growth hormone since the pituitaries from Groups 2 and 3 had significantly more growth hormone than the glands obtained from Group 1 ($P < 0.01$ in each case). Recent evidence (14) has shown that pituitary acidophil granulation does not necessarily reflect growth hormone content.

It was also found that neither the acidophil granulation nor growth hormone content of the pituitaries reflected the growth rate of the donor rat. After thyroidectomy there was a concurrent decline in the growth rate, pituitary growth hormone content and acidophil granulation. However, a comparison of the data from Groups 1, 2 and 4 showed that the body weights of Groups 2 and 4 were less than or equal to those of the untreated

FIG. 1. Anterior pituitary of a thyroidectomized rat. Note degranulation of the acidophils. ×840.

FIG. 2. Anterior pituitary of a thyroidectomized rat fed DNP in the diet for 18 days. Acidophils are degranulated. ×840.

FIG. 3. Anterior pituitary of an unoperated rat fed thyroid powder in the diet for 30 days. Note the decrease in the size and granulation of the acidophils. ×840.

FIG. 4. Anterior pituitary of a thyroidectomized rat treated with thyroxine for 33 days. Acidophil granulation has been restored. ×840.

FIG. 5. Anterior pituitary of an unoperated control to show the normal appearance of the acidophils. ×840.

thyroidectomized rats (Group 1), but that the pituitaries from thyroidectomized rats (Group 2) or unoperated rats (Group 4) fed DNP in the diet had significantly more somatotrophin than pituitaries obtained from thyroidectomized untreated animals. Indeed, the growth hormone content and structure of the glands from unoperated rats given DNP were not significantly different from those of intact controls.

In general, when the measure of somatotrophin was significantly decreased below normal, the pituitary acidophils were degranulated and the body weight was depressed. However, the decrease in pituitary growth hormone content could not be estimated on the basis of acidophil degranulation alone, and failure to maintain normal body weight was not necessarily accompanied by significant pituitary changes.

It appears that metabolic state, pituitary structure, and growth hormone content are interrelated in a complex manner which may influence the rate of secretion of somatotrophin.

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ISLET HYPERTROPHY IN OBESITY OF MICE BEARING ACTH-SECRETING TUMORS[1]

FRANZ X. HAUSBERGER AND ANDREW J. RAMSAY

Daniel Baugh Institute of Anatomy, Jefferson Medical College of Philadelphia, Philadelphia, Pennsylvania

ABSTRACT

The weight gain in mice bearing ACTH-secreting anterior pituitary tumors is caused by excessive growth of adipose tissue of normal composition. The islets of obese mice show hypertrophy, hyperplasia, and degranulation of beta cells, the degree of which is closely related to the extent of the obesity. The islet enlargement is caused by increase in size and number of beta cells. In severe adiposity, marked central cavitation of the islet was observed. Islet hypertrophy also develops, to a lesser degree, in animals maintained on restricted amounts of food. Despite weight loss, these animals are relatively fat. It is theorized that increased insulin secretion induces adipose tissue growth and obesity in these animals.

MICE bearing ACTH-secreting anterior pituitary tumors present features resembling those seen in Cushing's syndrome. Although morphological and functional changes in these animals have been extensively investigated (1–5) "the genesis of obesity in tumor-bearing mice as well as in Cushing's syndrome is obscure" (2). In the present study marked islet hypertrophy was found in these animals, the degree of which closely parallels the degree of adiposity. The possibility of increased secretion of insulin as the cause of the obestity is discussed.

METHODS

Donor mice bearing ACTH-secreting anterior pituitary tumors were supplied by Dr. Jacob Furth, to whom we are greatly indebted. The tumors were grafted into the thigh muscles of 45 female mice of the strain of origin (LAF_1 mice, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine). The body weight of the hosts (2 to 3 months old) was 20 to 24 gm. at the time of transplantation. Grafted animals and controls were weighed weekly during the first 4 to 8 weeks, and twice weekly after they had shown marked weight gain. All mice were fed Purina laboratory chow, most of them *ad libitum*. A small group of grafted animals and controls received limited amounts of food, sufficient to maintain their body weight or to induce slow weight loss. All animals of this restricted food group and six of the tumor-bearing mice and the controls with food *ad lib.* were analyzed (without liver) for body fat; nitrogen, fat and water content of the gonadal fat bodies were determined, using methods described elsewhere (6). The pancreas of all animals was stained by the method of Gomori (7).

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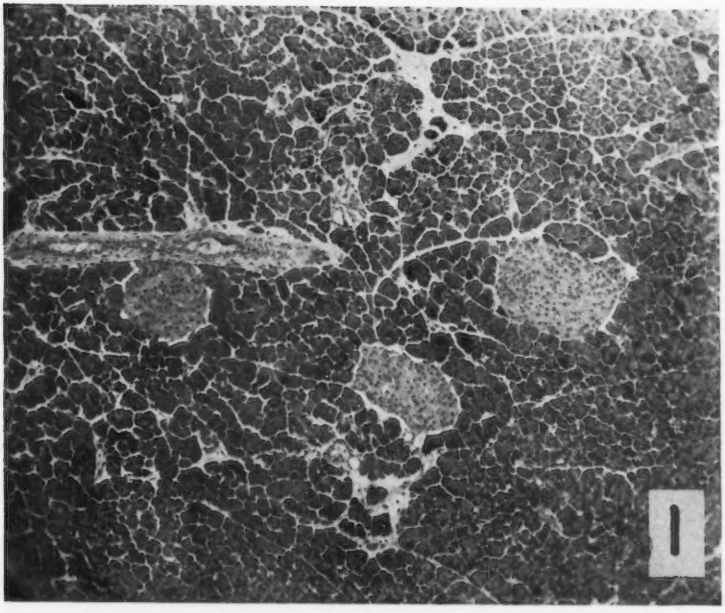
RESULTS

A. *Weight gain and body fat.* Almost all animals gained body weight in a rather uniform manner. For one to two months after transplantation the weight gain was normal, or sometimes slightly retarded, after which a pronounced continuous increase was observed for one to two months, occasionally up to four months. During this time, the tumor became palpable and visible. The body weight then remained at a plateau with only minor changes, usually from two to six weeks. Thereafter, a period of weight loss (often rapid) took place. The latter is due, according to Furth (1), to an overwhelming generalized fatal infection. At this time, the tumor frequently showed a diameter of more than two centimeters. Maximal body weights of 50 to 54 gm. were observed in about 10% of the animals 4 to 6 months after grafting. Two mice from this group were analyzed and showed, at a body weight of 50 and 54 gm., a fat content of 23.0 and 24.2 gm., respectively. Controls reached a body weight of 24 to 31 gm., with a maximum fat content of 3.9 gm. The amount of dissectable adipose tissue closely paralleled the amount of extractable body fat. Average protein, fat and water content of the ovarian fat bodies of normal controls was 1.1, 89, and 9.4%, respectively. The corresponding values for obese tumor-bearing mice were 0.9, 90, and 9%. The weight gain, therefore, is apparently caused by excess growth of adipose tissue of normal composition.

The degree of obesity was directly related neither to the size of the tumor nor to the time elapsing since the grafting took place. Generally, however, animals surviving for the longest time were the heaviest. Unfortunately, the weights of only a few of the markedly enlarged adrenals were recorded, therefore a possible relation between adrenal hypertrophy and obesity can not be established.

B. *Histology of the islets of Langerhans during periods of excessive weight gain.* Normally, most of the larger islets are found along the central artery of the dorsal pancreas (Fig. 1), while the islets of the more peripheral parts are smaller and less numerous. Accordingly, only corresponding areas of the pancreas of normal and obese mice were compared. The earliest and most persistent changes were degranulation of beta cells, slight enlargement of the islets, and a slight or moderate engorgement of the capillaries. Body weight of more than 35 gm. was always associated with islet hypertrophy (Fig. 2), which was caused by marked increase in the number and size of beta cells. Islets increased in number by new formation of beta cells from the duct system and centro-acinar cells, and possibly also from acinar cells (Fig. 3). This new formation was most obvious in the peripheral parts of the pancreas where normally fewer and smaller islets are found. The number of alpha cells was always markedly decreased, at least relatively. In many islets no recognizable alpha cells could be distinguished among the degranulated beta cells. Islets of giant size were found in all but one animal weighing 40 grams or more. Conservatively estimated, the islet volume of



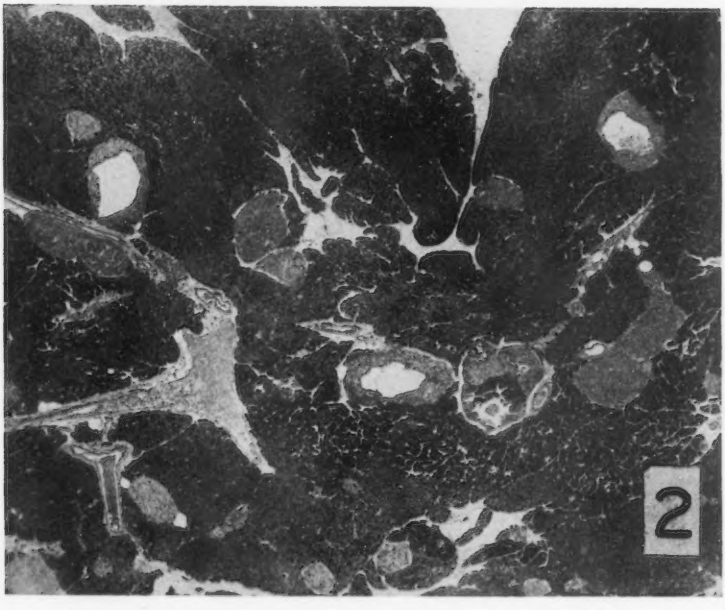


FIG. 1. Pancreas of a normal mouse, weighing 24 gm. Three medium-sized islets along the central artery are shown. On rare occasions, islets of approximately twice the size can be observed in normal animals. ×100.

FIG. 2. Pancreas of a tumor-bearing mouse, 4 months after grafting. The body weight increased during this time from 22 to 50 gm. Number and size of islets are greatly increased. Some islets show marked cavitation; almost all beta cells are degranulated ×30.

such severely obese mice exceeded that of the controls by 20 to 50 times. Many islets, especially the larger ones, showed extensive central cavitation. Fingerlike cords of beta cells were frequently seen extending into these cavities, which were filled with a lymph-like fluid, and, frequently, also with erythrocytes (Fig. 4).

C. *Islet changes during periods of weight loss* were similar to those seen in the heaviest animals, but the beta cells were of normal size and contained abundant amounts of granules. Islets of animals which had lost weight for several weeks showed normal numbers of alpha cells per islet.

D. *Histology of islets in tumor mice maintained on restricted food.* Tumor mice in the phase of rapid weight gain consume up to 50% more food than controls. In order to determine whether the increased food consumption induces the islet changes, the following experiment was performed. Eight young female mice were grafted and their body weights followed carefully. After six weeks, as in the controls, body weights had increased from 18 or 19 gm. to 23 or 24 gm. One tumor mouse, however, had shown a rapid weight increase in 28 gm. in the last week of this period. This sudden weight gain was considered a sign that the transplant had started to grow. At this time, five of these mice and three normal controls were transferred to individual cages and were fed 3 to 4 gm. of Purina laboratory chow daily. Three grafted mice and two normal controls received food *ad libitum*. The experiment was extended over a period of 30 days. The grafted mice with free access to food consumed about 7 gm. daily and increased their body weight to an average of 38 gm. At sacrifice, the tumors measured 6 to 8 mm. in diameter and the pancreas showed the typical changes already described. Normal controls fed *ad lib.* consumed 4.3 to 5.4. gm. daily.

The three non-grafted controls were maintained on 3 gm. of food per day and lost an average of 7% of their initial body weight. The islets showed a normal histological appearance. Three of the tumor-bearing mice received the same amount of food but lost 13, 25 and 32% weight, respectively. The other two grafted animals received up to 4 gm. of food daily, an amount which was sufficient to maintain the initial body weight in one animal while it induced in the other one a weight loss of 8%. At sacrifice, the tumors of many animals in this group were quite small. The islets of all animals were slightly to moderately enlarged and the beta cells showed minor de-

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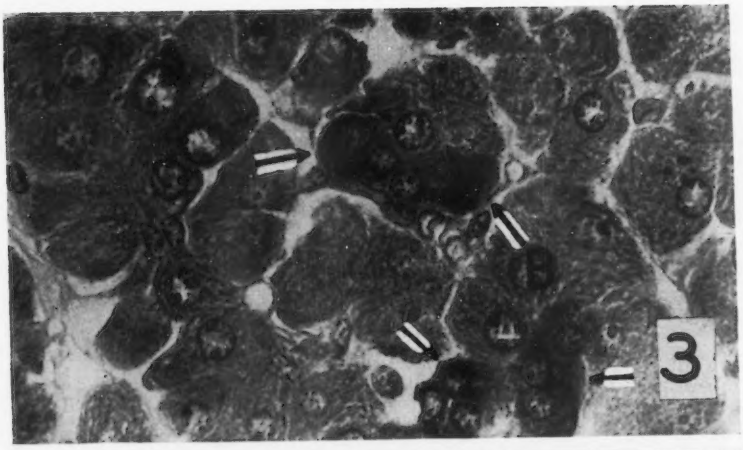
FIG. 3. Pancreatic acini of a tumor-bearing mouse. Two beta cells (B) within one acinus can be recognized due to their darker color. More beta cells are seen at the lower boarder of this photomicrograph. ×800.

FIG. 4. Photomicrograph of two islets of a tumor-bearing mouse, 5 months after grafting, weighing 42 gm. The cavity of the islet to the right is filled with erythrocytes which show no signs of clotting. ×100.

FIG. 5. Islet of tumor-bearing mouse maintained on restricted food for 30 days, during which period the body weight declined from 24 to 21 gm. A small cavity filled with blood can be seen within the enlarged islet. ×100.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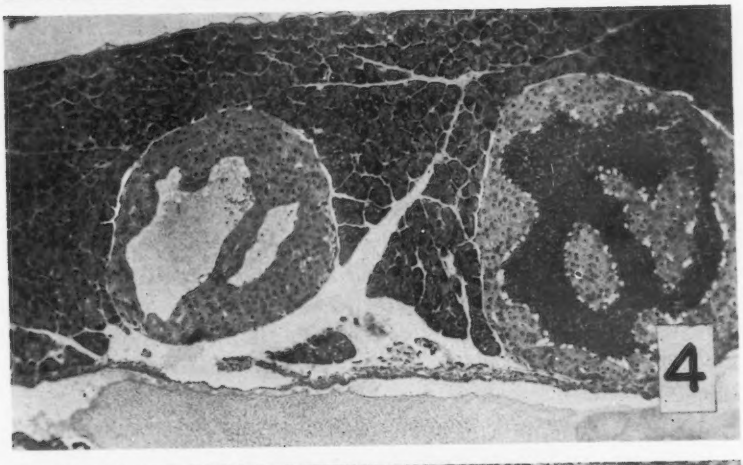
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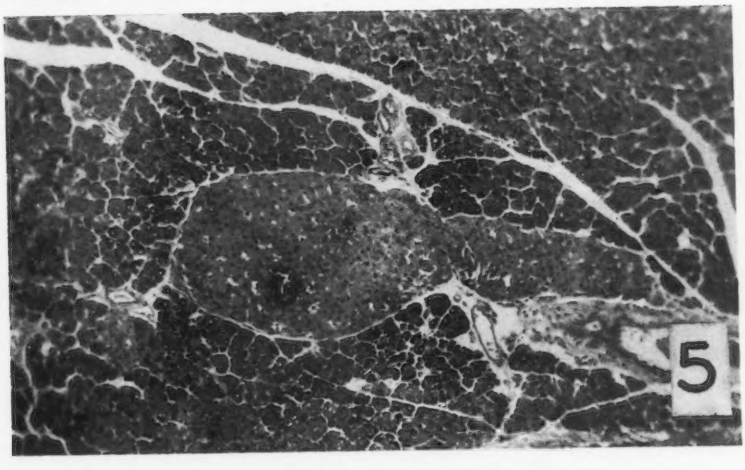
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granulation, the capillaries being slightly but distinctly dilated. The number and appearance of alpha cells seemed normal. All changes were closely related to the weight loss and were least marked in a mouse which lost almost one third of its weight. Minor islet cavitations filled with erythrocytes were found in one animal in this group (Fig. 5).

All animals appeared relatively fat at the time of sacrifice. Analysis of the whole animal, less liver, revealed a diminished amount of nitrogen but the fat content exceeded not only that of the controls maintained on restricted food but also that of normally fed mice of the same age. Detailed data will be presented at the completion of additional experiments.

E. *Histology of islets of adrenalectomized tumor mice.* Four mice were adrenalectomized, after grafting of the tumor, and maintained with Percorten. They gained weight at a slightly subnormal rate. At sacrifice, 4 months after the grafting, the large tumors showed a diameter of 1 to 1.5-cm, the fat content was normal (7.5 to 9.9% of body weight) and so was the histological picture of the pancreas.

DISCUSSION

Furth (1) and Furth *et al.* (2, 3, 4) have provided ample evidence that the pituitary tumor used in this investigation secretes only ACTH. The salient changes in the host are marked hypertrophy of the adrenal cortex, atrophy of the lymphatic organs, polyuria, obesity, increased nitrogen excretion and sometimes hyperglycemia and glycosuria. Reversal of these changes after adrenalectomy provides further evidence for ACTH-secretion. Sensitivity to infections frequently kills the host while the tumor is still small. There is no stimulation of any other endocrine gland but the adrenal. Extracts from lyophylized tumor tissue show adrenal ascorbic acid depleting activity, and the normal architecture of the adrenal cortex is maintained in hypophysectomized tumor-bearing mice. According to Wilson *et al.* (5), levels of blood corticosteroids, especially of corticosterone, are greatly increased.

It seems unlikely that tumor-induced metabolic changes other than ACTH-secretion and subsequent hyperadrenocorticism cause obesity and islet hypertrophy, since implanting of pellets of corticosterone and dehydrocorticosterone into mice of the same strain (LAF) produces comparable degrees of adiposity and islet changes (8). On the other hand, administration of these compounds to mildly alloxan diabetic LAF mice, without glucosuria and without weight loss, produced a severe diabetic condition, a rise of blood glucose levels from 145 mg% to 588 mg%, rapid loss of body weight and body fat and death (9). This observation is in general agreement with the known diabetogenic effect of the adrenal cortex.

The islet hypertrophy seen in hyperadrenocorticism seems to be caused neither by the increased food intake nor by the obesity. Severe hypothalamic obese rats show none (8) or only slight enlargement of the islets. Rats,

force fed for three weeks and gaining weight rapidly, show at the end of the experiment a fat content two to three times higher than controls but no noticeable hypertrophy of the islets (9). Furthermore, tumor mice maintained on restricted amounts of food also develop hypertrophy of the islets. These observations indicate that the tumor-induced hyperadrenocorticism stimulates islet hypertrophy which in turn suggests increased insulin secretion.

Direct proof for enhanced release of insulin, however, is missing, although several other observations tend to support this assumption. One of the major actions of insulin is on adipose tissue. It induces markedly enhanced fat deposition by stimulating true growth of that tissue in rats, and in rats receiving simultaneously large amounts of cortisone. Protein accumulation is depressed in these animals, but the excessive growth of fatty tissue is not significantly impaired (6). Insulin accelerates adipose tissue lipogenesis by acting on the fat cells directly. This effect continues as long as insulin is administered, whereas the enhancement of hepatic fat formation is only temporary. Pre-treatment with cortisone diminishes adipose tissue lipogenesis but insulin easily overcomes this effect (10). Insulin is also capable of markedly enhancing lipogenesis of adipose tissue (but not liver) in fasted rats (11). These results concerning the action of insulin on adipose tissue are in full agreement with the observation of relative obesity in our tumor-bearing mice receiving restricted amounts of food, and with findings by Zomzely and Mayer (12) who observed increased lipogenesis not only in tumor-bearing mice with free access to food but also in animals maintained on limited food intake.

All these observations, together with the observation of islet hypertrophy and hyperplasia suggest increased secretion of insulin as the direct cause of obesity in certain forms of hyperadrenocorticism.

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SOME EXPERIMENTAL DATA CONCERNING THE ROLE OF THE ADRENAL CORTEX IN THE SYNTHESIS OF LIVER GLUTATHIONE

ALEXANDRE ASCHKENASY AND GEORGES WELLERS*

Hôpital de la Pitié and Laboratoire de Physiologie de la Faculté de Médecine, Paris, France

ABSTRACT

In contrast to rats deprived of only one adrenal, bi-adrenalectomized rats submitted to a protein-free diet are incapable of synthesizing liver glutathione if they receive either cystine or glutamic acid alone. However, such a synthesis occurs after simultaneous administration of both amino acids or after supplementation with cortisone.

These observations suggest that glucocorticoids are necessary for mobilization of cystine or glutamic acid from the tissue protein stores.

AN INTERVENTION of the adrenals in the synthesis of glutathione has been suggested for many years (3) because of the finding of a high sulfhydryl concentration in these glands but it has subsequently been shown by more specific methods that the concentrations of glutathione were the same in the adrenals as in the kidneys and were even smaller than in the liver (4, 5). More recently, a decrease of reduced glutathione has been found in the blood of adrenalectomized rats (6), but injections of ACTH or cortisone seem to be without any effect on the blood levels of the tripeptide in normal dietary conditions (7, 8, 9, 10, 11).

In actual fact, a definite participation of the adrenal cortex in the synthesis of liver glutathione can be easily demonstrated in protein starved rats, but this intervention concerns only the delivery of free glutamic acid and cystine from tissue protein stores through the catabolic action of the glucocorticoid hormones.

Indeed, the chief purpose of our experiments was to verify the hypothesis that, in the absence of dietary proteins any neoformation of liver proteins or peptides should need the intervention of glucocorticoids for the delivery of amino acids from tissue stores and that therefore such a neoformation should be inhibited after the removal of adrenals. This would account for the inability of adrenalectomized rats to resist for more than a few days a protein deficient diet (12, 13) and also for the fact that supplementation of a protein deficient diet with methionine or cystine usually produces a relative hypertrophy of the liver if the rats are intact or deprived of only one adrenal but not if they are bi-adrenalectomized (14, 15).

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* Preliminary reports of this work have been published (1, 2).

In our experiments we attempted first to determine whether a peptide such as glutathione could be synthesized during protein starvation by bi-adrenalectomized as well as by uni-adrenalectomized control rats if these animals received either cystine or glutamic acid or both of them together.

We also investigated the question whether administration of cortisone was by itself sufficient to increase the liver glutathione levels in bi-adrenalectomized protein starved rats, or if at least the hormone would facilitate in such rats the utilization of extra cystine for the synthesis of the tripeptide.

MATERIALS AND METHODS

One hundred nineteen male albino rats (Wistar) weighing about 200 gm. were divided into 17 groups. The animals of the three first groups were, as controls, subjected to the removal of the left adrenal only. The rats of the other 14 groups were bi-adrenalectomized (under ether anesthesia). The latter received to drink a solution of 1% NaCl.

All animals, housed in individual cages, were immediately after the operation submitted for the period of a week to a diet free of proteins, of the following composition (per 100 gm. of dry food): sucrose 79.3 gm.; white dextrin 10 gm.; peanut oil 5 gm.; salt mixture (n° 22, Orent-Keiles) 5.7 gm.; ascorbic acid 25 mg.; thiamine 1 mg.; riboflavine 2 mg.; niacine 20 mg.; pyridoxine 2 mg.; calcium pantothenate 3 mg.; folic acid 100 μg.; vitamin B_{12} 5 μg.; mesoinositol 60 mg.; D-biotine 20 μg.; tocopherol 1 mg.; vitamin K 1 mg.; choline chlorhydrate 500 mg.; two drops of a solution of vitamins A (50.000 i.u. per cc.) and D (10.000 i.u. per cc.). The dry food was mixed with 50% water. The diet was given *ad libitum*.

All animals were killed with ether on the eighth day. Immediately after death, a fragment of the liver was put into a 10% solution of trichloracetic acid for a determination of reduced and total glutathione, performed by the method of Binet and Weller (12, 13). The wet and dry weight of the whole liver was calculated by adding together the weights of the precedently removed and the remaining fragments, the latter being weighed before and after drying at 100° C. Statistical analyses were carried out by means of the "t"-test.

First experiment

This experiment was designed to study the action of cystine and glutamic acid on liver glutathione in uni-adrenalectomized controls and in bi-adrenalectomized rats, both submitted to a protein-free diet.

Among the control rats, one group (12 rats) received daily 1 cc. water by stomach tube, another group (6 rats), 50 mg. L-cystine* in 1 cc. water by the same route, the third group (6 rats), 61 mg. L-glutamic acid,* also by intubation.

Among the bi-adrenalectomized rats (6 groups), the first group (12 rats) was given daily only 1 cc. water by stomach tube; the second group (11 rats), 50 mg. L-cystine, the third group (6 rats), 61 mg. L-glutamic acid, the fourth group (6 rats), concurrently 50 mg. cystine and 31 mg. glycine, the fifth group (9 rats), 50 mg. cystine and 61 mg. glutamic acid; and finally the sixth group (8 rats) received the three amino acids together at the same doses as before, but in 2 cc. (instead of 1 cc.) water.

Second experiment

The purpose of this experiment was to investigate whether administration of adequate doses of cortisone would render protein starved bi-adrenalectomized rats as sensitive

* Hoffmann-La Roche.

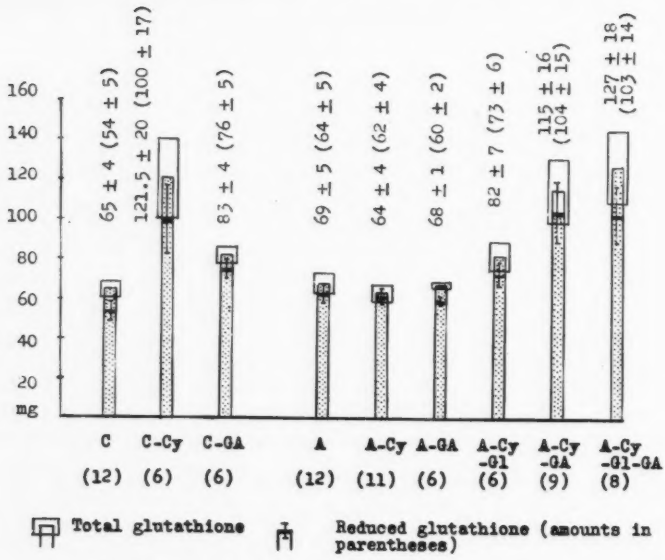

FIG. 1. Amounts of liver glutathione (mg. per 100 gm. of liver tissue, ± S.E.M.) in control rats (deprived of one adrenal) (C) and in bi-adrenalectomized rats (A). All animals have been deprived of proteins for 7 days. Effects of administration of cystine (Cy), glutamic acid (GA), cystine+glycine (Cy–Gl), cystine+glutamic acid (Cy–GA) and finally, cystine+glycine+glutamic acid (Cy–Gl–GA). The numbers of rats are indicated in parentheses below the column of each group.

to extra cystine as are uni-adrenalectomized controls concerning the utilization of this amino acid for the synthesis of glutathione.

The study was performed on 8 groups of adrenalectomized animals. The two first groups (5 and 6 rats) received 50 μg. per day cortisone (acetate); the two following ones (5 and 4 rats), 75 μg.; two other groups (5 rats in each of them), 200 μg.; finally the two last groups (8 and 5 rats), 1 mg. cortisone. The second, forth, sixth and eighth groups were also supplemented with cystine (50 mg. per day). Cortisone and cystine were given by stomach tube in 1 cc. water.

As in the preceding experiments, the animals were killed after one week of protein starvation and the weight of the liver as well as the concentration of glutathione in this organ were determined.

RESULTS

First experiment (Fig. 1)

1. The liver of the bi-adrenalectomized rats contained more reduced glutathione than the liver of the controls but the amounts of total glutathione were similar in both groups if the animals were not supplemented with any amino acid.

2. Administration of cystine alone produced a noticeable increase of glutathione (reduced as well as oxidized) in control rats ($P < 0.05$) whereas it did not change the amounts of the tripeptide in the bi-adrenalectomized

rats. The difference between the effects of cystine in controls and in adrenal-deprived rats appeared still greater if the amounts of glutathione were calculated not per 100 gm. of liver tissue but for the whole liver. Indeed, cystine increased the relative (wet and dry) weight of this organ (per 100 gm. of body weight) in control rats (wet weight: 4,757 mg. ±133* with cystine; 4,281 mg. ±83* without cystine) while it did not modify it in bi-adrenalectomized rats (3,705 mg. ±107* with cystine; 3,588 mg. ±77* without cystine).

3. Glutamic acid alone was even in control rats much less effective than cystine. Nevertheless it too induced in these rats a significant increase of liver glutathione ($P < 0.01$). Like cystine, glutamic acid was without any effect in bi-adrenalectomized rats.

4. Simultaneous administration of cystine and glycine to adrenal-deprived rats was not much more efficacious than cystine alone

5. The addition of glutamic acid to cystine elicited a striking increase of glutathione in bi-adrenalectomized rats and the difference from the levels found after cystine alone was highly significant ($P < 0.01$).

6. The amounts of liver glutathione in bi-adrenalectomized rats given concurrently cystine, glutamic acid and glycine were not higher than those found after both cystine and glutamic acid.

Second experiment (Fig. 2)

1. In rats not supplemented with cystine, cortisone given at doses of 50 μg. or 75 μg. per day did not change the amounts of liver glutathione in comparison with those found in adrenalectomized non-treated rats (compare figures 1 and 2).

On the contrary, doses of 200 μg. and 1 mg. cortisone induced by themselves, without any amino acid supplementation, an important increase of liver glutathione, of the reduced as well as of the oxidized form.

2. Extra cystine did not elicit any additional increase in rats receiving 200 μg. or 1 mg. cortisone, but with smaller doses of cortisone (especially with 50 μg. per day), the same amino acid increased significantly ($P < 0.05$) the synthesis of glutathione as it did in uni-adrenalectomized controls.

DISCUSSION

It is well known that in normal rats administration of cystine alone is sufficient to increase the amount of liver glutathione if the diet is entirely or partly deprived of proteins (18, 19, 20).

A priori there may be two explanations for the lack of glutathione synthesis after giving cystine (or glutamic acid) alone to protein-starved rats if the latter are deprived of both adrenals: 1) the inability of these animals to link together cystine (cysteine), glutamic acid and glycine; 2) their inability to deliver one or another of these amino-acids from the tissue protein stores.

* Standard error of mean.

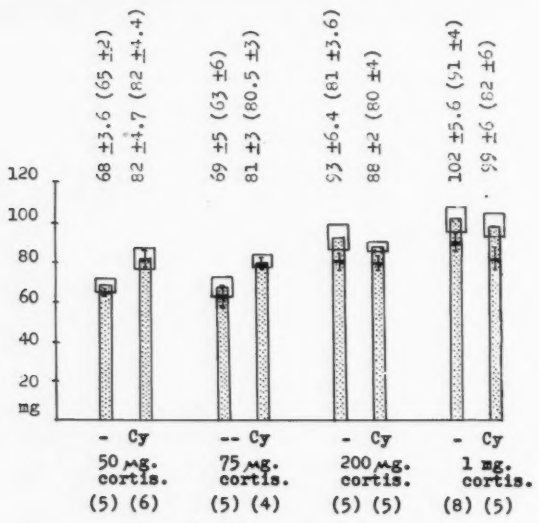

FIG. 2. Amounts of total and reduced glutathione of the liver (mg. per 100 gm. of liver tissue) in adrenalectomized protein-deprived rats given different doses of cortisone. Effects of extra-cystine. The numbers of rats are indicated in parentheses below each column.

Only the second explanation is to be considered since a striking increase in liver glutathione occurs as soon as one gives the three amino-acids together, or even only both cystine and glutamic acid. The lack of delivery and production seems to concern glutamic acid as well as cystine for neither simultaneous administration of cystine and glycine nor that of glutamic acid alone is efficacious.

The deficient production of glutathione might be one of the reasons for the high mortality noticed in adrenalectomized rats during a diet very poor in proteins (12, 13). Indeed the survival of these protein-deprived rats is not at all influenced by administration of cystine alone, whereas it increases significantly if one gives concurrently cystine, glycine and glutamic acid (13).

The ability of cortisone to mobilize tissue proteins and to thus deliver free amino acids might explain the high amounts of glutathione in the liver of bi-adrenalectomized protein-deprived rats given 200 µg or 1 mg. cortisone per day, though these rats were not supplemented with any amino acid. Smaller doses of cortisone (50 µg. or 75 µg. per day) seem to be barely adequate to permit a sufficient production of free glutamic acid but not that of cystine. This would explain why such doses do not increase by themselves the levels of liver glutathione but induce this effect if the rats receive extra cystine.

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THE EFFECT OF PROPYLTHIOURACIL ON THE CONVERSION OF MONOIODOTYROSINE TO DIIODOTYROSINE[1]

D. WARD SLINGERLAND, DOROTHY E. GRAHAM, ROMA K. JOSEPHS, PHILIP F. MULVEY, JR., ANNE P. TRAKAS, AND EIICHI YAMAZAKI

Radioisotope and Medical Services, Veterans Administration Hospital and Tufts University School of Medicine, Boston, Massachusetts

ABSTRACT

The ratio of monoiodotyrosine to diiodotyrosine (MIT/DIT) and triiodothyronine to thyroxine (T_3/T_4) was increased by feeding rats propylthiouracil before the injection of I^{131}. This change occurred even when the uptake of I^{131} by the thyroid had returned to normal by allowing 24 hours or more to elapse after stopping propylthiouracil or by using small amounts of propylthiouracil. A depletion of total iodine in the gland was not the cause since the MIT/DIT remained elevated when total iodine was preserved by adding thyroid to propylthiouracil. A high thyrotropin did not account for the result since the added thyroid would have suppressed this. A high intrathyroidal iodide was not the explanation since the addition of KSCN made no difference. Thyroid alone increased MIT/DIT probably by inhibiting the thyrotropin essential to the conversion of monoiodotyrosine to diiodotyrosine. The results indicated that the conversion of monoiodotyrosine to diiodotyrosine was more sensitive to propylthiouracil than the iodination of tyrosine to form monoiodotyrosine. A block of the conversion of monoiodotyrosine to diiodotyrosine by propylthiouracil with a resultant increase in triiodothyronine relative to thyroxine, would help to account for the observed high radioiodine uptakes in hyperthyroid patients made euthyroid by propylthiouracil.

CERTAIN antithyroid compounds inhibit the formation of organic iodine substances but not the accumulation of iodide ions by the thyroid (1) and thus interfere in the synthesis of thyroid hormone between iodide ions and iodinated tyrosyl radicles. The coupling of acetyldiiodotyrosine to form acetylthyroxine has been inhibited by antithyroid compounds *in vitro* (2) but there is, as yet, no evidence that these compounds have an effect on the coupling of diiodotyrosine *in vivo* (3).

The present study, undertaken to determine whether antithryoid compounds have an effect on a step in the chemical synthesis of thyroxine other than the iodination of tyrosine, led to the conclusion that propylthiouracil inhibits the conversion of monoiodotyrosine to diiodotyrosine. The reaction

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of monoiodotyrosine with iodine to form diiodotyrosine is more sensitive to propylthiouracil than the combination of iodine with tyrosyl radicles to form monoiodotyrosine.

METHODS

Sprague-Dawley rats (150–200 gm.) were fed diets of Ken-L-Biskit (supplemented with iodide to contain 20 μg. per 10 gm. of diet) to which were added propylthiouracil (0.1% to 0.00001%), thyroid (0.1 to 0.2%), iodide (20 μg. to 1 mg. per 10 gm. diet), potassium thiocyanate (2 mg. per 10 gm. diet), and sulfanilamide (2.5%). In some instances, thyrotropin (Armour Thytropar 5 to 10 I. U. per day) was injected intraperitoneally. The animals were fed these diets for from 1–11 days; the diets were discontinued (unless otherwise noted) 24 hours before the injection of I^{131} (20–100 μc) and the animals were then fed the control diet. Twenty-four hours after the injection of radioiodine the thyroids were removed and hydrolyzed with pancreatin in Krebs-Ringer-phosphate medium with added propylthiouracil at pH 7.4 for 24 hours. Aliquots (8–20 μl) were placed on paper chromatographic strips. The solvents used were: butanol-acetic acid water (75–10–15), butanol-ethanol-2N NH_4OH (5–1–2), and t-amyl alcohol saturated with 2N NH_4OH. The strips were radioautographed, counted by a strip counter (Nuclear-Chicago) and the areas under the curves measured by planimeter. The ratios referred to in the text and tables (such as MIT/DIT) were determined by using the figures for these areas directly from the planimeter readings. No corrections were made for diiodotyrosine and thyroxine containing more iodine than monoiodotyrosine and triiodothyronine. The uptake of radioiodine by the control animals was less than 10% (8–10%) and all other uptakes are expressed as a percentage of the control.

The thyroids were usually grouped in the tubes for hydrolysis and then counted. After aliquots were removed for chromatography, the thyroids were analyzed for chemical iodine by a modified Barker method (4). The specific activity of the components was determined by chromatographing 75 μl and then cutting out the appropriate areas of the paper for measurements of radioactive and chemical iodine.

RESULTS

Effects of Propylthiouracil

The ratio of radio-monoidotyrosine to radio-diiodotyrosine (MIT/DIT) was 0.48 in the control rats and 2.2 in the propylthiouracil (PTU) fed animals (Table 1). Radiomonoiodotyrosine represented 22% in the control

TABLE 1. THE EFFECT OF PROPYLTHIOURACIL AND PROPYLTHIOURACIL PLUS THYROID HORMONE ON THE RATIO OF RADIOMONOIODOTYROSINE TO RADIODIIODOTYROSINE AND RADIOTRIIODOTHYRONINE TO RADIOTHYROXINE

Diet	Days*	Expts.	Rats	Thyroid weight (mg.)	MIT/DIT	T_3/T_4	Uptake	λ
KLB20**	4–10	17	68	15	0.48 (0.12–0.7)	0.21 (0.09–0.4)	100	9.0 5.7–16.4)
KLB20+PTU (0.1%)	1–10	19	76	22	2.2 (0.85(1)–3.3)	1.6 (0.38(3)–3.9)	38 (5–140)	2.4 (1.2– 4.1)
KLB20+PTU+TH (0.2%)	4	5	20	15	1.5 (0.57(2)–2.1)	V.H.	11 (7–13)	7.2 (5.8– 9.4)

* In all these experiments the diets were changed to KLB20 twenty-four hours before I^{131} injection.

** KLB20 = Ken-L-Biskit with iodide added (20 μg./10 gm. diet). All determinations were made on groups of 4 rats. λ = total μg. of I^{127}/thyroid. V.H. = very high; T_4 was usually unmeasurable so that the ratio was infinite. See text for discussion. The figures in parenthesis represent the extremes. (1) The 0.85 was the only experiment below 1.3. (2) The 0.57 was the only experiment below 1.3. (3) The 0.38 was the only experiment below 1.2. Uptake = the radioactivity of the glands counted in a well counter and expressed as the % of the control (100).

TABLE 2. THE EFFECT OF PROPYLTHIOURACIL ON THE RATIO OF RADIOMONOIODOTYROSINE TO RADIODIIODOTYROSINE AND CHEMICAL MONOIODOTYROSINE TO CHEMICAL DIIODOTYROSINE

Diet	Days	Rats	Thy. wt. (mg.)	MIT^{131}/DIT^{131}	MIT^{127}/DIT^{127}	MIT^{131}/MIT^{127}	DIT^{131}/DIT^{127}	Uptake	λ
KLB20	4	4	15	0.38	0.33	0.1314	0.1117	100	9.5
KLB20+PTU (0.1%)	4	4	27	2.2	2.0	0.6396	0.5790	90	1.9
KLB20+PTU* 0.1%)	4	4	26	12.8	1.2	0.0756	0.0071	12	0.8
				T_3^{131}/T_4^{131}	T_3^{127}/T_4^{127}	T_3^{131}/T_3^{127}	T_4^{131}/T_4^{127}	Uptake	
KLB20	4	4	15	0.29	—	—	—	100	9.5
KLB20+PTU (0.1%)	4	4	27	3.5	2.8	0.1709	0.1357	90	1.9
KLB20+PTU* (0.1%)	4	4	26	9.2	2.2	0.0097	0.0024	12	0.8

* Diet continued until sacrifice resulting in such a high degree of block in binding by PTU that little radioiodine gets through, leading to a deficiency of radioiodine for combination with tyrosine and an extremely high MIT/DIT (see beginning of Discussion for amplification). That this does not apply to I^{127} is indicated by the MIT^{127}/DIT^{127} being increased to the same degree as that for rats whose uptake is back to normal (second group with 90% uptake). There may be a small additional increase in MIT^{131}/DIT^{131} because higher levels of PTU (when the diet is not stopped) more effectively block the conversion of MIT to DIT. The data, however, does not prove this.

and 43% in the treated animals of the total radioactivity in the thyroid. The ratio of radio-triiodothyronine to radio-thyroxine (T_3/T_4) was 0.21 in the controls rats and 1.6 in the treated animals (Table 1). These figures represent the average of about 20 experiments in which the rats were fed the test diets for 4 days and were then fed the control diet for one day before the injection of radioiodine. The chemical iodine ratio of monoiodotyrosine to diiodotyrosine, though not identical to the radioiodine ratio, indicated that a similar change had occurred (Table 2). The total chemical iodine in the gland was reduced from an average of 9 μg in the control to 2.4 μg. in the treated animals and the gland size approximately doubled (1.66×normal). The preponderance of monoiodotyrosine lasted for 72–96 hours after stopping propylthiouracil. The results were similar whether the experiment was run for 10 days or for only 1 day, although the monoiodotyrosine to diiodotyrosine ratio was not elevated as much by 1 day of treatment. A single injection of 5 mg. of propylthiouracil also raised the ratio of mono-

TABLE 3. THE EFFECT OF DECREASING QUANTITIES OF PROPYLTHIOURACIL ON THE RATIO OF RADIOMONOIODOTYROSINE TO RADIODIIODOTYROSINE

Diet*	Days	Rats	Thyroid wt. (mg.)	MIT/DIT	Uptake	λ
KLB20	4	4	14	0.41	100	4.1
KLB20+PTU (0.1%)	4	4	24	15	76	1.0
KLB+PTU** (0.001%)	4	4	22	0.75	307**	2.1
KLB+PTU** (0.0001%)	4	4	13	0.8	299**	3.9
KLB+PTU** (0.00001%)	4	4	15	0.48	810**	17.0[1]

* In all groups the diets were continued until sacrifice; I^{131} was given 24 hours before sacrifice.

** These animals received a much smaller amount of iodide (about 2–5 μg./10 gm. diet) and thus the uptakes are rather high compared to control. Other experiments, however, indicate that low levels of PTU (0.00001% or less) occasionally result in a high uptake of radioiodine (Table 4). Repeated experiments do not prove this to be a consistent finding, although further study is being done.

[1] This high λ is not satisfactorily explained. This was not a consistently repeatable finding with low levels of PTU and on occasion a low λ (Table 4) or a normal λ was found. Contamination would have to be minimal to result in such a small increment of iodine.

TABLE 4. THE EFFECT OF SMALL DOSES OF PROPYLTHIOURACIL IN STIMULATING RADIOIODINE UPTAKE

Diet*	Days	Rats	Thyroid wt.	MIT/DIT	Uptake	λ
			mg.			
KLB20	4	4	15	0.21	100	10
KLB20+PTU (0.01%)	4	4	21	7.3	35	1.2
KLB20+PTU (0.001%)	4	4	16	2.9	60	1.6
KLB20+PTU (0.0005%)	4	4	16	3.3	110	3.5
KLB20+PTU (0.0001)%	4	4	15	0.9	115	4.0
KLB20+PTU (0.00001%)	4	4	15	1.3	266	2.5

* All diets were continued until sacrifice; I^{131} was injected 7 hours before sacrifice.

iodotyrosine to diiodotyrosine which returned to normal 48–72 hours after the injection. If the diets were continued until the time of sacrifice, the monoiodotyrosine to diiodotyrosine ratio was increased up to 15 fold by 0.1% propylthiouracil but was normal, in some instances, only when the propylthiouracil was reduced below 0.001% to 0.00001% (Table 3, 4).

The average uptake of radioiodine at 24 hours was reduced to 38% of normal (about 10% of the administered dose) when 0.1% propylthiouracil was given for 4 days and discontinued 1 day before the injection of I^{131}. In many instances with individual rats, however, the uptake was normal, and the increase in MIT/DIT still occurred. When 48 hours elapsed after stopping propylthiouracil, the uptake was normal but MIT/DIT was still increased. When a low level of propylthiouracil (0.0005–.00001% in the diet) was fed for four days and maintained for the 24 hours after the injection of I^{131}, the uptake of radioiodine was actually increased, in some instances, without an increase in inorganic radioiodide (Fig. 1).

Effects of Thyroid Hormone

The addition of 0.2% thyroid to the control diet did not change the ratios of monoiodotyrosine to diiodotyrosine or triiodothyronine to thyroxine (Table 5). The 24 hour uptake of radioiodine was reduced to 15% and there

TABLE 5. THE EFFECT OF TH ON THE RATIO OF RADIOMONOIODOTYROSINE TO RADIODIIODOTYROSINE

Diet	Days	Expts	Rats	MIT/DIT	T_3/T_4	Uptake	λ
KLB20	4–10	17	68	0.48 (0.12–0.7)	0.21 (0.09–0.4)	100	9.0 (5.7–16.4)
KLB20+TH	4–10	4	16	0.4 (0.28–0.63)	<1.0*	15 (2–25)	12.0 (10–13)

* Difficult to quantitate accurately due to low radioactivity. <1.0 is not necessarily >0.21. These results were based on a visual estimation of radioautography.

KLB20	4	1	4	0.26	<1.0**	100	7.5
KLB20+TH*	4	1	4	2.0	<1.0**	10	7.5

* The diet was continued until sacrifice.
** Difficult to quantitate due to low radioactivity.

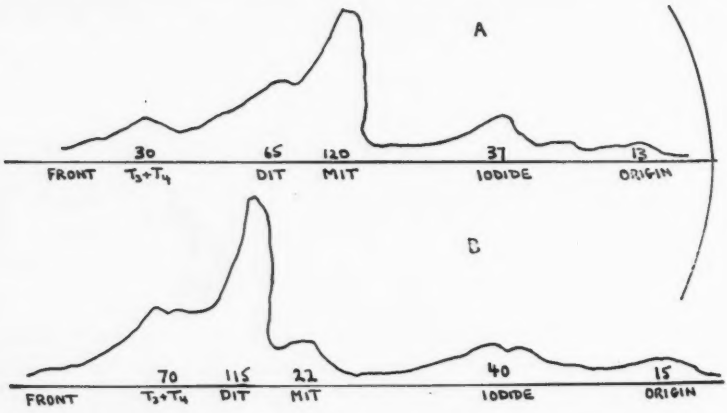

FIG. 1. Effect of a low dose of propylthiouracil (0.0005%), continued until sacrifice, on the various iodinated compounds in the thyroid. A is the count of the chromatographic strip of the pooled thyroids of four rats on propylthiouracil 0.0005%, and B the control. Solvent: butanol-acetic acid-water (75–10–15). The curved line represents the ordinate. MIT = monoiodotyrosine; DIT = diiodotyrosine; T_3 = triiodothyronine; T_4 = thyroxine. T_3 and T_4 are not separated in this solvent system and include a significant amount of unknown radioactivity. The figures represent the areas under the curve as measured by planimeter. Note that A has a normal iodide, a normal total content of radioactivity, but a high MIT/DIT.

was an increase in total gland iodine to 12 μg. When thyroid was added to propylthiouracil, the MIT/DIT remained elevated and T_3/T_4 increased further (Table 1). The addition of thyroid reduced the radioactivity to such an extent that thyroxine could not be counted. It would seem probable, however, that the ratio probably stayed the same and the apparent increase in T_3/T_4 was due to the insensitivity of the counter to the very small amount of radiothyroxine present. The uptake of radioiodine was reduced from 38% to 11% while the total gland iodine was returned toward normal (7.2 μg.) and, in individual experiments, to normal.

It is of interest that, when TH alone was continued through the experiment until sacrifice, there was a pronounced increase in MIT/DIT (Table 5).

Effects of Potassium Iodide

The addition of potassium iodide to the control diet (1 mg./10 gm.) did not change MIT/DIT and T_3/T_4 on the average (Table 6) although there were 2 experiments (one of eleven days and one of four days) out of 25 in which MIT/DIT increased and 3 (all of four days) in which T_3/T_4 increased. These exceptions could not be consistently repeated nor explained. The uptake of radioiodine at 24 hours was 50% of normal and the total gland iodine was normal. In one experiment, iodide was given for 36 hours,

TABLE 6. THE EFFECTS OF IODIDE (KI 1 MG./10 GM. DIET) ON THE RATIO OF RADIOMONOIODOTYROSINE TO RADIODIIODOTYROSINE

Diet	Days	Expts.	Rats	MIT/DIT	T_3/T_4	% Uptake	λ
KLB20	4–10	17	68	0.48	0.21	100	9.0
KLB20+KI	1–10	25	96	0.48 (0.13–1.8)	<1.0*	50 (20–92)	9.3 (6.9–13.7)
KLB20	$1\frac{1}{2}$	1	4	0.32	—	100	5.7
KLB20+KI*	$1\frac{1}{2}$	1	4	0.15	—	12	4.6
KLB20	3	1	4	0.42	0.43	100	7.0
KLB20+PTU	3	1	4	2.2	3.3	82	3.0
KLB20+PTU+KI	3	1	4	2.8	—	39	3.0
KLB20+KI+TH	4–10	6	24	0.67	—*	7.5	8.8
KLB20	4	1	4	0.27	<1.0*	100	9.0
KLB20+PTU	4	1	4	1.0	>1.0	89	2.4
KLB20+TH+KI	4	1	4	1.2	>1.0	7	7.0

* Due to low specific activity T_3 and T_4 were difficult to quantitate. These results were based on visual estimation of radioautography. <1.0 is not necessarily >0.21. There seemed to be no significant change.

* KI was continued until sacrifice. T_3 and T_4 could not be measured.

* T_3/T_4 was impossible to quantitate with consistency.

* T_4 and T_3 were difficult to quantitate. These results were based on visual estimation or radioautography.

after the first 12 hours of which I^{131} was injected, again without an effect (Table 6). The addition of iodide to propylthiouracil did not change the elevated MIT/DIT (Table 6). When iodide was added to thyroid the uptake of radioiodine was reduced to 7.5% but there were no other differences from the control (Table 6). Potassium iodide was added to propylthiouracil and thyroid without additional change in MIT/DIT or T_3/T_4 (Table 6). Smaller amounts of carrier iodide were added to the control diet (20–100 μg./10 gm.) or to the radioiodine (150 μg.) with no difference in the results. Animals were fed a moderately low iodine diet (about 2.5 μg./gm.) for 4 days and for 30 days and one group was given water alone for 3 days without change in MIT/DIT.

Effect of Potassium Thiocyanate

The addition of potassium thiocyanate (2 mg./10 gm. of diet) to the control diet did not change MIT/DIT or T_3/T_4. When thiocyanate was given to animals receiving propylthiouracil, MIT/DIT and T_3/T_4 remained elevated (Table 7).

Effects of Thyrotropin

The injection of thyrotropin (5 USP units/day) into the animals on a control diet did not change MIT/DIT or T_3/T_4. When the animals receiv-

TABLE 7. THE EFFECTS OF THIOCYANATE, TSH AND SULFANILAMIDE ON THE RATIO OF RADIOMONOIODOTYROSINE TO RADIODIIODOTYROSINE

Diet	Days	Rats	Thy. Wt.	MIT/DIT	Uptake	λ
KLB20	4	3	—	0.44	—	—
KLB20+KSCN*	4	3	—	0.58	—	—
* 2 mg. KSCN/10 gms. diet.						
KLB20	3	4	19.5	0.42	100	7.0
KLB20+PTU	3	4	19.5	2.2	82	3.0
KLB20+PTU+KSCN	3	4	23.5	1.6	70	2.0
KLB20	3	4	16	0.12	100	7.5
KLB20+PTU+TH	3	4	15	2.4	8	8.0
KLB20+PTU+TH+TSH*	3	4	13.5	2.5	16	3.5
* 5 USP Units every day for 3 days.						
KLB20	4	4	15	0.27	100	5.2
KLB20+Sulfanilamide* (2.5%)	4	4	18.5*	0.36	450	2.5
* Thyroid weight (for all 4) was increased from the control of 59 to 74 but in another experiment there was no goiter produced and the uptake was increased to 200% of normal.						
KLB20	4	4	15	0.21	100	10
KLB20+SULFA (2.5%)*	4	4	18.5	2.6	59	3.2
* Diet was continued until sacrifice. This experiment was different in that the I^{131} was injected only 7 hours before sacrifice. The increase in MIT/DIT may be an effect similar to PTU, although further work is required to establish whether this is a direct effect of the drug.						
KLB20	3	2	16	0.22	100	—
KLB20+TSH*	3	2	14	0.31	130	—
* 5 USP units IP every day for 3 days. The last injection was 24 hours before I^{131}.						

ing propylthiouracil and thyroid were injected with thyrotropin, there was no further change in MIT/DIT or T_3/T_4 (Table 7).

Effects of Sulfanilamide

The addition of sulfanilamide (2.5%) to the control diet did not change MIT/DIT or T_3/T_4. The twenty-four hour uptake was increased over normal, even when goiters were not produced (Table 7). When sulfanilamide was continued until sacrifice, however, goiter was produced, uptake was diminished and MIT/DIT was increased.

DISCUSSION

When tyrosine (0.01 M in an ammoniacal solution) is iodinated *in vitro*, the number of iodine atoms per tyrosine molecule determines the relative amounts of monoiodotyrosine and diiodotyrosine formed (5). A peak quantity of monoiodotyrosine occurs with 3 iodine atoms whereas nothing but diiodotyrosine is formed when an excess (6 atoms) of iodine is available. It has been reported that iodine deficiency leads to the formation, *in vivo*,

of more monoiodotyrosine than diiodotyrosine (6, 7, 8) and more triiodothyronine relative to thyroxine (6, 7). These results were obtained using very low iodine diets (10–40 μg./kg.) for long periods of time (3–12 months).

It is therefore possible that, in the present study, an incomplete block of binding imposed by propylthiouracil might have allowed a limited amount of iodine to become available for tyrosyl iodination, leading to a preponderance of monoiodotyrosine. In many instances, however, the uptake of radioiodine at twenty-four hours by the animals fed propylthiouracil was equal to or greater than that by the controls and MIT/DIT was still increased. Further evidence indicating that often there was no block in binding included a normal amount of iodide and a normal or greater than normal amount of organic iodine compounds (Fig. 1) in the thyroid. This indicates that, in spite of the organification of an amount of radioiodine, and presumably chemical iodine,[2] at least equal to the controls, the propylthiouracil fed animal made less diiodotyrosine relative to monoiodotyrosine.

Attempts to influence further the ratio of monoiodotyrosine to diiodotyrosine, by changing the amount of iodine bound, failed. This included varying the dietary iodine (up to 1 mg./10 gm. diet), injections of thyrotropin, adding thyroid to the diet, and adding potassium thiocyanate to the diet. Finally, an increase in MIT/DIT occurred while rats were still being fed a small amount (0.00001%) of propylthiouracil in spite of an increase in the uptake of radioiodine (Table 4).

For a number of reasons, then, the high MIT/DIT cannot be due merely to a partial block of binding which persisted for an interval after propylthiouracil was stopped: (1) the observation that uptakes were often normal (2) there was often no increase in inorganic iodide to suggest a block in binding (3) further reduction in uptake did not lead to a greater increase in MIT/DIT (4) attempts to increase the amount of iodine going through a partial block failed to diminish MIT/DIT (5) iodide, which also blocks binding for 26–42 hours, failed to elevate MIT/DIT,[3] and finally (6) small

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[2] In a single experiment urine specific activities were determined. Specific activity of the urine of the control rats was 0.41 and of the urine of the rats fed propylthiouracil was 0.44.

[3] Galton and Pitt-Rivers (*Endocrinology* **64**: 835. 1959) have recently shown an increase in MIT/DIT for 8–16 hours following a single injection of 500 μg. iodide, during which time a block in binding occurred. Wolff *et al.* (*Endocrinology* **45**: 504. 1949) indicated that 500 μg. iodide injected every 8 hours maintained a block in binding for 26, and in a single rat, for 50 hours after the first injection. These animals would presumably have had a high MIT/DIT on the basis of iodine deficiency. Our failure to find an increase in MIT/DIT at 36 hours could be due to the variation in iodide intake (the iodide was added to the diet, not injected), the longer time (24 hours) after radioiodine before sacrifice, or chance variation resulting in a shorter period of block in binding.

levels of propylthiouracil, below the level where a block in binding could be detected, still raised MIT/DIT.

It is of interest that, when thyroid was continued through to the end of the experiment, there was an increase in MIT/DIT. This suggests that one can effectively achieve the results of hypophysectomy (9) in increasing MIT/DIT by suppressing thyrotropin with thyroid. When thyroid was discontinued 24 hours before I^{131} was given, MIT/DIT had usually returned to normal, probably because of a rapid restoration of uninhibited secretion of thyrotropin. The exceptions were presumably due to a more prolonged suppression of thyrotropin after thyroid had been stopped.

The thyroids of PTU fed animals are depleted of iodine. The evidence (10, 11) that the total iodine content of the thyroid exerts an influence on the iodide concentrating mechanism suggests the possibility that a low intrathyroidal iodine content might decrease or increase the rates of other reactions resulting in an increased MIT/DIT. The maintenance of total iodine in the gland by the addition of thyroid to propylthiouracil, however, did not decrease the elevated MIT/DIT.

The ratio of monoiodotyrosine to diiodotyrosine in the thyroglobulin of hypophysectomized rats is above normal and is returned to normal by the injection of thyrotropin (9). Propylthiouracil, fed to rats in adequate amounts, stimulates the release of thyrotropin which could effect the rates of various reactions in the iodination of thyroglobulin resulting in changes in the relative amounts of various compounds. The addition of thyroid to propylthiouracil however, did not decrease the elevated MIT/DIT. Neither the injection of thyrotropin alone nor the administration of thyrotropin to rats fed propylthiouracil changed MIT/DIT. Thyrotropin is not responsible, therefore, for the observed changes in MIT/DIT.

As intrathyroidal concentrations of iodide block the iodination of thyroglobulin (12) and as administration of propylthiouracil leads to an increased content of iodide in the thyroid it could be argued that the rates of other reactions in addition to the iodination of thyroglobulin might be affected. However, potassium thiocyanate, when added to propylthiouracil, did not change MIT/DIT. Moreover, in many instances, particularly with low doses of propylthiouracil, there was no evidence, chromatographically, for a high intrathyroidal iodide. The effect of propylthiouracil on MIT/DIT, then, was not caused by a high intrathyroidal iodide content.

Some of the various reactions in the synthesis of thyroid hormone seem to be independently affected not only by TSH (9) but also by antithyroid compounds. The evidence presented suggests that in addition to blocking the iodination of thyroglobulin propylthiouracil also inhibits the conversion of monoiodotyrosine to diiodotyrosine. In the presence of an adequate amount of monoiodotyrosine but a limited amount of diiodotyrosine, the synthesis of triiodothyronine is relatively facilitated over that of thyrox-

ine and the ratio of triiodothyronine to thyroxine is increased. It is difficult to assess, on the basis of this study, any other possible effects of propylthiouracil on thyroxine synthesis, such as the coupling of diiodotyrosine radicles to form thyroxine, since an increase, for example, in the ratio of diiodotyrosine to thyroxine could be secondary to the preferential synthesis of triiodothyronine in the presence of high MIT/DIT. It is clear, however, that the conversion of monoiodotyrosine to diiodotyrosine is more sensitive to propylthiouracil than the conversion of iodide to iodinated tyrosyl radicles.

The observation of Halmi (13) that the addition of perchlorate (or thiocyanate) to propylthiouracil resulted in the formation of monoiodotyrosine (in addition to iodide alone) but no other organic iodine compounds (14) may be explained by perchlorate partially inhibiting the block of propylthiouracil on iodine binding but not interfering with the much more effective block of propylthiouracil between monoiodotyrosine and diiodotyrosine. It has been suggested that perchlorate acts on an iodide-thiouracil reaction (14), presumably assisting in the oxidation of iodide. It was suggested that a different enzyme system is involved in the addition of the second iodine to tyrosine than in the addition of the first. Supportive evidence included the inability of tissue homogenates (15) and foetal thyroid at a certain stage (16), and the limited ability of certain non-toxic nodules (17) to form diiodotyrosine from monoiodotyrosine. The possibility of a chemical explanation, namely, that the second iodine combines with tyrosine less readily than the first, remains, though the present study does not favor it over the former possible explanation.

The observation, in thyrotoxicosis, of a continuing high 24 hour uptake of bound radioiodine (18) during the administration of propylthiouracil could be explained at least partially by a block, after iodination of tyrosine, between monoiodotyrosine and diiodotyrosine. The change during propylthiouracil therapy in the relative amounts of T_4 and T_3 which are synthesized would lead to the PBI being deceptively low, since a greater proportion of the thyroid hormone would consist of T_3. If these effects are to occur, it must be assumed that, during the course of PTU treatment, the concentration of the drug in thyroid tissue may drop low enough so that a significant amount of binding of iodine by the thyroid can occur.

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ADRENOCORTICAL, SPLENIC AND REPRODUCTIVE RESPONSES OF MICE TO INANITION AND GROUPING[1]

J. J. CHRISTIAN

Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland

ABSTRACT

Male mice respond to being placed in groups with increased adrenocortical and decreased reproductive activity as a result of social competition. Experiments were performed to determine whether food, either limited in amount or localized in source, would increase competition, and thereby increase the degree of adrenal hypertrophy and reproductive suppression resulting from grouping. Male mice in groups of either 4 or 5 were provided food in four different ways: *ad lib* scattered; *ad lib* from a single feeder which could be defended by one mouse; limited in amount and scattered; and limited in amount from a feeder. In one experiment (2 replications) the quantitative limitation was 4.0 gm. of food per mouse per day, which was less than the daily *ad lib* intake but still permitted a gain in weight. In a second experiment food was restricted to 3.5 gm. per mouse per day, a restriction which resulted in a weight loss during the experimental period. Appropriate segregated controls were used. After one week the following weights were collected: body, adrenals, thymus, spleen, preputials, testes, and seminal vesicles. Grouping resulted in adrenal and splenic hypertrophy, and diminished weights of the reproductive organs. The location or amount of food was without effect on the adrenals, but inanition resulted in a decrease in the splenic response to grouping. Quantitative food restriction resulted in a significant decrease in the weights of the reproductive organs, especially of the sex accessories. It was concluded that (1) social competition among grouped mice was not increased by limiting the supply of food in amount, location, or both; (2) inanition is not a stimulus to increased pituitary-adrenocortical activity, and (3) inanition acts directly to decrease the activity of the reproductive system and not indirectly as a result of stress.

THE present investigations were conducted to explore the relationship between inanition and adrenocortical activity; and to determine the effects of food restriction on the adrenal, thymic, splenic, and reproductive responses to social competition between mice.

There is still some question about the ability of acute or chronic inanition to stimulate pituitary-adrenocortical activity (1, 2). The literature on the subject is somewhat contradictory. Guinea pigs respond to inanition with true cortical hypertrophy (3). Rats apparently respond to total star-

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[1] The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

vation with cortical hypertrophy (3, 4), but this change was shown to be due to increased fluid content rather than true hyperplasia (3). Chronic inanition does not result in adrenal hypertrophy in rats and there may be atrophy associated with a decrease in the width of the *zona fasciculata* (3, 4, 5). Baker concluded that the peripheral changes in rats associated with chronic inanition do not result from a generalized reaction to stress (5). Hamster adrenals decrease in weight with starvation, but there may be increased secretory activity of the cortex (6). The adrenals of mice do not change in weight or ascorbic acid content as a result of 35 days on 60% of the normal daily *ad lib.* diet (7), although there is an increase in relative adrenal weight due to a marked loss in body weight (7, 8). It does not seem appropriate to use relative weights when the experimental procedure results in a marked loss in body weight even though it may be argued that the failure of the adrenals to lose weight with a loss in body weight may itself represent a kind of hypertrophy.

Nevertheless, food restriction markedly suppresses the reproductive system, especially the sex accessories, of guinea pigs, mice, rats and other species (2, 3, 4, 5, 9, 10).

Inanition or starvation apparently acts to reduce the production of pituitary gonadotrophins without affecting the production of ACTH (2, 11–14). One of the purposes of the present experiments is to provide additional clarification on the ability of chronic inanition to elicit a generalized stress response in mice.

Adrenocortical hypertrophy, thymic involution, and diminished reproductive activity occur when previously segregated mice are placed in groups (14–16). The degree of adrenocortical hypertrophy and of reproductive suppression is related to the number of animals constituting a group and the social structure within the group (18). These responses occur in the presence of abundant food and water and with all other environmental necessities maintained as optimally as laboratory conditions permit. The degree of adrenocortical hypertrophy found in a given individual generally is related inversely to its rank in a social hierarchy (17, 18).

Clarke (19) and Chitty (20) describe splenic hypertrophy in voles as a result of social competition. We wished to extend this finding to house mice in these experiments.

Definitive experiments elucidating the relative roles of crowding and of a limited food supply, the latter acting either directly on the individual or indirectly as a competitive factor, or both, have not so far been performed. Increased competition, if present, should result in increased adrenocortical activity and decreased reproductive function (cf. above). The present experiments were designed to clarify these relationships and to answer the following specific questions: (1) is competition for food, whether limited in amount or abundant, significant among grouped mice, (2) does competition for food become significant if it is available only from a single source so

that one dominant animal might restrict its availability to others; (3) what are the effects of moderately restricted and frankly deficient food supplies on the pituitary, adrenocortical and reproductive systems; and finally, (4) how do the responses to the abundance and location of food modify the responses of grouping?

MATERIAL AND METHODS

Two experiments were performed using the same general procedure. They were designed to take advantage of the power of analyses of variance to examine the data and determine the relative contributions made by the several experimental variables in eliciting changes in the adrenals, thymus glands, spleens, testes and accessory reproductive organs of albino male mice.

Male mice were segregated in individual cages at weaning (3 weeks of age; 8–10 gm.). Three weeks later they were selected at random and either placed in groups of 4 or 5 mice each or were left segregated to serve as controls. A week after grouping, the animals were weighed and sacrified. The following organs were fixed and weighed as described elsewhere (13): adrenals, thymus glands, spleen, testes, paired seminal vesicles (full) with coagulating glands, and preputial glands.

The food used was a complete diet in the form of small pellets and manufactured by Dietrich and Gambrill of Frederick, Maryland.

In each experiment food was supplied in four ways: two groups received food in abundance and well scattered over the floor of the cage; two groups received abundant food but available only from a single source; the remaining four groups were given a limited amount of food daily and any remaining from the preceding day was removed and weighed prior to supplying a fresh amount. The limited food was scattered for two of the latter groups and placed in a feeder for the remaining two. The single source of food consisted of a metal tube one inch in diameter with a grilled bottom, thus a dominant mouse, if he so desired, could deny the other mice access to the food. The segregated controls received either a limited supply of food or an amount in excess of usage, but the food was scattered for all.

In the first experiment the groups consisted of four mice each and the quantity of food was restricted to 4.0 gms. per mouse per day, an amount determined to be slightly less than the average daily *ad lib* consumption per mouse. This experiment was repeated once in its entirety. These two replications are noted subsequently as replication A and B of experiment I.

In the second experiment the food limitation was 3.5 gm. per mouse per day and the groups consisted of five mice each (see table).

Except as otherwise noted, the data were examined by analyses of variance. Since two groups of segregated mice, one with scattered food and one with food in a feeder, were not established, two analyses were necessary for each organ in each experimental arrangement. One analysis included grouped and segregated mice. The variances produced by grouping as opposed to segregation, by the amount of food provided, and by different experiments were determined. A second analysis was used for the grouped mice only. The contributions made to the total variance for each measurement by the location of the food, the amount of food and by different experiments were determined with this analysis. In no instance did the results of the two analyses differ from each other when the same factor was examined in both. Finally, all of the data from all of the experiments were combined and again examined using three analyses, one with the grouped animals only, a second with grouped and isolated animals and a third with only the segregated animals (Table 1) as described above. Measurements on individual mice were used as the units of measurement throughout the statistical treatment rather than

TABLE 1. RESULTS OF ANALYSES OF VARIANCE OF THE DATA SHOWING THE EFFECTS OF GROUPING, LIMITED FOOD SUPPLY AND RESTRICTED SOURCE OF FOOD ON THE ADRENALS, THYMUS, SPLEEN AND REPRODUCTIVE ORGANS OF MALE ALBINO MICE**

			Change in body wt. gm.		Adrenals mg.		Thymus mg.		Spleen mg.		Preputials mg.		Testes mg.		Seminal Vesicles mg.	
			Mean	P diff.	Mean	P diff.	Mean	P diff.	Mean	P diff.	Mean	P diff.	Mean	P diff.	Mean	P diff.
Exp. I Food Limitation: 4.0 gm./day/mouse Groups of 4 mice each	Food Source†	scattered	+2.22	*	5.89	*	41.6	*	124.7	*	96.2	*	213.5	*	112.2	*
		feeder	+2.48		6.18		48.1		107.4		104.8		206.4		120.4	
	Food Amount	unlimited	+3.17	<0.0001	5.92	*	45.1	*	104.0	*	109.7	*	216.9	*	137.5	<0.01
		limited	+1.55		5.87		46.7		104.9		101.7		209.7		116.2	
	Caging	grouped	+2.35	*	6.04	<0.05	44.8	*	116.0	<0.01	100.5	<0.05	209.9	*	116.3	<0.001
		segregated	+2.38		5.62		47.8		83.8		114.9		219.2		145.6	
	Replication	A	+3.21	<0.0001	5.72	<0.05	38.3	<0.001	83.8		116.2	<0.002	225.1	<0.001	145.3	<0.001
		B	+1.44		6.08		54.1		102.5	*	94.3		200.5		106.8	
									106.6							
Exp. II Food Limitation: 3.5 gm./day/mouse Groups of 5 mice each	Food Source†	scattered	+0.01	*	6.45	*	50.2	*	120.3	*	92.0	*	191.0	*	92.9	*
		feeder	−0.62		6.10		56.6		101.5		87.3		192.7		78.2	
	Food Amount	unlimited	+1.60	<0.0001	6.04	*	49.8	*	110.9	<0.01	103.5	<0.001	201.1	<0.05	114.9	<0.001
		limited	−1.88		6.07		49.8		87.1		80.1		185.8		79.2	
	Caging	groups of 5	−0.31	*	6.27	<0.05	53.5	<0.02	110.6	<0.001	83.6	*	191.9	*	85.4	<0.002
		segregated	+0.22		5.58		42 6		75.7		95.6		196.2		118.8	
Experiments I & II Data combined: Segregated and grouped mice	Food Source†	scattered			6.10	*	44.8	*	123.1	*	94.7	*	205.1	*	105.0	*
		feeder			6.15		51.3		105.1		98.1		201.2		104.2	
	Food Amount	unlimited			5.96	*	46.8	*	106.5	*	107.4	<0.01	211.1	<0.05	129.2	<0.001
		limited			5.94		47.9		98.3		93.6		200.8		102.3	
	Caging	grouped			6.12	<0.01	48 1	*	114.0	<0 001	96.4	<0.05	203.1	*	104.6	<0.001
		segregated			5.61		45.9		83.9		108.0		211.0		136.1	
	Expts.	I, 4/group 4.0 gm. food			5.89	*	45.9	*	104.4	*	105.7	<0.01	213.3	<0.001	126.9	<0.001
		II, 5/group 3.5 gm. food			6.06		49.8		98.8		91.6		193.3		96.7	
Segregated mice only	Food Amount	unlimited	+2.56	<0.001	5.45	*	46.2	*	89.8	<0.01	118.4	<0.02	221.0	<0.05	150.5	<0.01
		limited	−0.77		5.74		45.7		72.1		97.6		201.0		121.6	
	Expt.	I (4.0 gm./day+unlim.)	+1.88	<0.02	5.62	*	47.8	*	83.8	*.	114.9	<0.05	219.2	<0.01	145.6	<0.05
		II (3.5 gm./day+unlim.)	+0.11		5.58		42.6		75.7		95.6		196.2		118.8	

† Analyses of data from grouped mice only. Other analyses included grouped and segregated mice except where specifically noted otherwise.
* Not significant at 95% confidence level.
** Standard errors of the means in this table are not given, as these means are the combined results of several experiments in each case. Therefore standard errors are not appropriate.

the mean value for each group, as the basic relationship between grouping and the responses to it has been established (14–16).

RESULTS

Food consumption and body weight

Mice given 4.0 gm. of food per mouse per day gained weight during the experimental 7-day period at an average daily rate of 0.22 of a gm. compared to 0.45 of a gm. for those fed *ad lib* ($P < 0.001$) (Table 1). The average daily food consumption was 3.86 gm. per mouse for those restricted to 4.0 gm. per day with some groups not consuming all of the provided food. Food remained 11 times and was completely consumed 17 times of the total 28 group days by the grouped mice on a restricted diet. The average *ad lib.* food consumption was 4.47 gm. per mouse per day. Four grams per day, therefore, represented a moderate restriction of food which served to decrease the average gain in weight during the experimental period. The source of food, whether scattered or in a feeder, was without effect on body weight (Table 1). No difference in body weight was seen with respect to whether the mice were grouped or segregated (Table 1).

Segregated and grouped mice receiving 3.5 gm. of food each per day always completely consumed the daily food supply and each lost an average of 0.27 gm. daily while their controls fed *ad lib.* each gained an average of 0.27 gm. per day ($P < 0.001$) (Table 1). This amount of food was insufficient to maintain growth and therefore constituted a deficient food supply. In spite of the food restriction the source of food had no effect on body weight. Similarly grouping had no appreciable effect on body weight. In no group did a single animal gain weight by virtue of dominating the food supply while all of the others in the same group lost weight. All of the animals on a limited amount of food per day lost weight. There was no more than a gram difference between the heaviest and lightest mice in these two groups. It is evident from these experiments that body weight was unaffected by any of the experimental variables other than the actual amount of food provided.

Adrenal weight

Adrenal weight was affected significantly only by the manner of caging (Table 1). The adrenals of grouped mice averaged 9.7% heavier than those of segregated mice ($P < 0.01$). Adrenal weight was unaffected by the source of food, whether scattered or in feeders. Inanition did not produce adrenal hypertrophy or atrophy.

Thymus weight

There were no significant variations in thymus weight associated with the experimental procedures except in the one experiment in which food was limited to 3.5 gm. per mouse per day and the mice were in groups of

five each (Table 1). In this experiment the thymus weights of grouped mice were significantly greater ($P < 0.02$) than those of isolated mice, which could represent diminished or delayed involution due to decreased output of androgens. The weights of the accessory reproductive organs would suggest such an event. The results of earlier experiments support such a conclusion (5). Similar changes in thymus weight with grouping were not observed in the two experiments with four mice per group.

Splenic weights

Grouping resulted in a 41% increase in splenic weight ($P < 0.001$) when the data from all experiments were combined. This was also true for each of the individual experiments (Table 1). Food restriction to 3.5 gm. per mouse per day produced 22% decrease in splenic weight; but splenic weight was unaffected by restricting food to 4.0 gm. per day.

Preputial glands

The preputial glands of grouped mice weighed an average of 10.7% less than those of segregated mice when the data from the three experiments were combined ($P < 0.05$). Preputials of mice restricted to 3.5 gm. of food a day weighed 21.5% less than their controls ($P < 0.001$) (Table 1). Furthermore the preputials of grouped animals restricted to 3.5 gm. per day weighed 11.4% less than those of grouped animals restricted to 4.0 gm. per day ($P < 0.01$), while the perputials of segregated mice weighed 32.9% less on 3.5 gm. than on 4.0 gm. of food per day.

Group size, whether 4 or 5, did not significantly affect preputial weight in these experiments. The difference in preputial weight between grouped and segregated mice was significant with groups of four each, but was not significant in experiments with groups of five each (Table 1). The lack of significance in the latter case probably results from confounding the analysis by limiting food to 3.5 gm. per day since the preputial weights of segregated mice and of mice in groups of five on unlimited food are compared, it is clear that there was a significant decrease (17.5%; $P < 0.02$) in preputial weight, but to a lesser extent than food restriction under these conditions.

Seminal vesicles

The responses of the seminal vesicles to grouping and food restriction were much more marked than those of the preputial glands (Table 1). The seminal vesicles of mice from groups of four weighed significantly more than those of mice from groups of five when food was supplied *ad lib.* The seminal vesicles were the most sensitive to the experimental variables of all of the organs weighed, but were unaffected by differences in the source of food.

Testicular weight

The weights of the testes were not significantly decreased by grouping (Table 1), but were decreased significantly by a deficient food supply (Table 1). The source of food did not affect testicular weight. The effects on testicular weight of limited food supply and grouping were similar to but less marked than those on the preputial glands and seminal vesicles.

Differences between experiments

The data in Table 1 show that there were significant variations between replications and between experiments. Nevertheless further examination (Table 1) will show that differences between control and experimental animals were comparable throughout; so that results and conclusions are not affected by these variations.

DISCUSSION

The design of these experiments permitted examination of the effects of a variety of variables in caging and food supply on the pituitary-adrenocortical and reproductive systems of male mice. If food, either limited in amount or restricted in its location, increased competition between mice, there should have been increases in adrenal and splenic weight in the grouped animals subjected to these alterations in food supply. Such increases in weight did not occur, even when the food supply was deficient. Splenic weight was decreased when food was limited to 3.5 gm. but not when it was limited to 4.0 gm./mouse/day. Therefore, a restricted food intake affected splenic weight in some way other than through a generalized stress response.

Grouping resulted in a significant hypertrophy of the adrenals and spleen, in contrast to inanition, but had little effect on the thymus. The splenic hypertrophy was appreciable, confirming Chitty's results with voles (20).

The thymus glands of albino mice are unresponsive to grouping as shown by these and earlier experiments (14, 16). One might expect to find a decline in thymic weight with grouping and increased adrenocortical activity. Thymic involution resulting from social stress is clearly evident in brown house mice (14, 16) or voles (18–20), but these animals are conspicuously more reactive and exhibit more pronounced adrenal responses than do the albino mice (14, 16).

Grouping also appreciably diminished the weights of the preputial glands and seminal vesicles, although there was no significant change in mean testicular weight. The decrease in the weights of the preputials and seminal vesicles is consistent with results of earlier experiments (13–15) and suggests a diminished production of androgens even though the decrease in testicular weight with grouping was not significant.

The results discussed indicate that grouping may induce generalized responses to stress in mice and that these responses are unaffected by the amount or location of the food supply used in this experiment. It therefore seems that the effects produced by grouping may result from purely sociophysiological factors and that competition for food need not increase the amount of social interaction in the group over the levels existing as a result of the grouping itself.

Food location, whether scattered or available from a single source, was without effect on the reproductive organs. However, the testes, preputials, and seminal vesicles were adversely affected when the daily food supply was limited in amount. The experimental design permitted assessing separately the effects of food supply and grouping on organ weight. In these experiments quantitatively limited food supply affected the reproductive organs more adversely than did grouping. A restricted amount of food affected the reproductive organs more adversely than did grouping, and did so without affecting the adrenals and spleen, whereas grouping affected the adrenals, spleen, and reproductive organs to comparable degrees. Finally, a deficient food supply (3.5 gm. per day per mouse) had a much more marked effect on the reproductive organs than did a limited, but adequate, supply (4.0 gm. per mouse per day), although both had significant effects. A diminished amount of food evidently does not affect mice by evoking a generalized response to stress. A restricted food intake apparently affects the reproductive organs of male mice without eliciting a generalized stress response, probably by affecting the secretion of gonadotrophins (2, 11, 12, 13, 14). These results are in agreement with Baker's conclusion that inanition does not increase adrenocortical activity in rats (5).

Splenic weight was significantly decreased by limiting the food per mouse to 3.5 gm. per day, a reversal of the increase in weight produced by grouping. Food deficiency evidently affects the spleen, as it does the reproductive organs, without producing a generalized stress response.

It is concluded that moderate inanition does not stimulate increased pituitary-adrenocortical activity in mice and that it may affect the reproductive system independently of the pituitary-adrenocortical system, possibly by diminishing the secretion of gonadotrophins (2, 11, 12). It is further concluded that moderate limitation of food, in either source or amount, or both, does not necessarily increase competition between mice. Extreme limitation may result in increased interaction.

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THE EFFECTS OF PROPYLTHIOURACIL AND PERCHLORATE ON THE BIOGENESIS OF THYROID HORMONE[1]

JOHN B. RICHARDS AND SIDNEY H. INGBAR

Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts, and the Howard Hughes Medical Institute, Miami, Florida

ABSTRACT

Graded doses of propylthiouracil (PTU), administered to rats 30 minutes prior to tracer doses of I^{131} and 270 minutes prior to sacrifice, resulted in progressive reductions in total I^{131} uptake, I^{131} organic-uptake, and alterations in the relative proportions of the radioiodinated amino acids. Progressive blockade was associated with an increasing proportion of MIT, and a decreasing proportion of DIT and total thyronines. Comparable reductions in thyroidal I^{131} organic-uptake induced by perchlorate were not attended by alterations in relative composition of the radioiodinated amino acids. Administration of PTU to rats previously treated with TSH resulted in alterations in radioiodinated amino acid composition similar to those observed with PTU alone, despite a normal I^{131} organic-uptake. The effects of PTU cannot therefore be ascribed to the reduction in the flux of iodine into organic moieties. Triiodothyronine was detected in less than half of the glands from treatment-free controls and in none of the glands from PTU-treated rats in which a reduction in thyroxine formation occurred.

These results indicate that PTU affects not only the initial oxidation of iodine and the resulting monoiodination of tyrosine, but also inhibits both the diiodination of tyrosyl residues and their coupling to form the hormonally active iodothyronines.

The findings provide a possible explanation for the restoration of a eumetabolic state in patients with Graves' disease receiving PTU, whose I^{131} uptakes remain abnormally high.

THE biogenesis of thryoid hormone may be considered a three stage process. First, the thyroid gland concentrates inorganic iodide from the extracellular fluid, thus providing a concentrated substrate for subsequent reactions within the gland. Next, iodide is oxidized, presumably to elemental iodine, which then iodinates tyrosyl residues to form first monoiodo- and then diiodotyrosine. Finally, the iodotyrosines undergo coupling to form the iodothyronines, including thyroxine and triiodothyronine.

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It has been clearly established that the antithyroid agents of the thioureylene group exert their goitrogenic action in rats and their ameliorative effects in human thyrotoxicosis by interfering with the glandular production of physiologically active hormone (1). These agents do not interfere with the glandular capacity to concentrate inorganic iodide. However, when administered in large doses, they decrease or virtually abolish the ability of the thyroid gland to incorporate intrathyroidal iodide into organic moieties (2, 3). For this reason, it seems likely that these agents interfere with the conversion of inorganic iodine to a more highly oxidized form capable of iodinating tyrosine within thyroidal protein.

The present studies were undertaken in order to elucidate the observation, made in this laboratory (4) and elsewhere (5), that patients with Graves' disease may continue to demonstrate increased thyroidal uptakes of radioiodine while receiving quantities of antithyroid medication adequate to restore their metabolic status to normal or below. In such patients, the majority of accumulated radioiodine cannot be discharged from the gland by thiocyanate and, therefore, is presumed to be bound in organic form. In view of these findings, it seemed probable that the antithyroid drug not only diminishes the total quantity but also alters the chemical nature of the organically-bound iodine within the gland, favoring the synthesis of hormonally inactive products.

To assess this hypothesis, rats have been given graded doses of propylthiouracil (PTU). Effects upon both total glandular accumulation of iodine and the nature of the iodinated amino acids produced have been observed. Qualitative changes in iodinated amino acids induced by PTU might result either from 1) a specific effect of this agent on stages of hormonogenesis subsequent to the initial oxidation of iodine, or from 2) a reduction in the total flux of iodine into organic moieties. Therefore, comparable studies were performed to assess the effects of perchlorate, an agent thought to exert its antithyroid action solely by decreasing the capacity of the gland to concentrate inorganic iodide (6). It has been found that in doses which produce comparable reductions in organic iodinations within the gland, perchlorate and PTU exert profoundly different effects upon the nature of the iodinated amino acids formed. The present studies indicate that PTU affects not only the initial oxidation of iodine and the resulting mono-iodination of tyrosine, but also inhibits both the di-iodination of tyrosyl residues and their coupling to form the hormonally active iodothyronines.

METHODS

Male Sprague-Dawley rats of uniform weight were maintained on Purina Laboratory Chow and water *ad libitum* prior to and during experimental manipulations.

Three or four animals comprised each experimental group. All injected materials were administered intraperitoneally in 1.0 ml. of a solution in isotonic sodium chloride. Doses of the drugs employed are listed in Table 1.

A total of thirty-one rats were employed as controls in nine experiments. Each rat received 5 μc. of carrier-free I^{131} four hours prior to sacrifice.

TABLE 1. EFFECT OF ANTITHYROID DRUGS ON TOTAL I^{131} UPTAKE, I^{131} ORGANIC-UPTAKE AND RELATIVE PROPORTION OF RADIOIODINATED AMINO ACIDS IN RAT THYROID GLANDS

Dose of Drug		No. Rats	4-Hr. Total I^{131} Uptake (% dose/rat thyroid)	4-Hr. I^{131} Organic-Uptake (% dose/rat thyroid)	% Glandular Radioiodinated Amino Acids		
(mg./rat)	(mg./kg.)				MIT	DIT	T_4
			Controls				
0	0	31	2.6±0.4*	2.3±0.4	33.3±3.1	53.0±3.4	13.5±4.0
			Propylthiouracil				
0.01†	0.05	4	1.09	0.96	34.5	54.5	11.1
0.03	0.08	4	0.99	0.86	52.8	44.4	2.8
0.05	0.19	6	0.51	0.44	59.6	38.9	1.5
0.10	0.43	4	0.27	0.14	66.3	33.7	0‡
0.20	0.82	4	0.19	0.09	70.4	29.6	0
0.40	1.36	4	0.30	0.12	73.4	26.6	0
0.80	2.35	4	0.23	0.07	71.4	28.6	0
1.60	4.36	4	0.09	0.02	77.3	22.7	0
3.20	18.00	4	0.11	0.01	78.6	21.4	0
			Perchlorate				
0.3	1.7	3	0.53	0.46	39.6	47.1	13.3
0.3	1.7	4	0.48	0.43	30.8	56.6	12.6
1.5	7.4	4	0.11	0.10	34.9	56.0	9.0
4.5	20.0	4	0.03	0.03	42.8	52.8	4.3
45.0	216.0	4	0.009	0.008	44.4	47.7	7.9
45.0	260.0	3	0.006	0.005	43.9	43.9	12.2
			TSH + Propylthiouracil				
0	0	4	2.66	2.30	35.5	51.4	13.1
0 +TSH	0	4	6.38	5.50	35.5	53.2	11.3
0.01+TSH	0.04	4	4.23	3.70	42.4	49.2	8.5
0.05+TSH	0.16	4	2.02	1.60	55.6	44.4	0
0.10+TSH	0.36	4	0.61	0.30	64.5	35.5	0

* Standard deviation for 9 groups of rats (3–4 rats per group).

† This dose administered 30 min. prior to I^{131} and again 90 min. after injection of the tracer.

‡ Zero indicates compound detected on neither scan nor radioautograph of chromatogram.

Experimental groups received antithyroid drugs and I^{131} according to the following dosage schedule:

0 minutes: antithyroid drug
30 minutes: I^{131}
120 minutes: antithyroid drug
270 minutes: sacrifice

In one experiment, each of sixteen rats was given TSH (Thytropar, Armour) in a dose of 0.6 units subcutaneously at 12-hour intervals for a total of five doses. Twelve hours following the final injection of TSH, the animals were divided into four groups. Three groups of TSH-treated rats were given doses of PTU (Table 1) according to the schedule noted above, and one group was kept as TSH-treated controls. In addition, four animals were studied as treatment-free controls.

Assay of Thyroidal Radioiodine Uptake: Rats were sacrified by a blow on the head and thyroid glands were quickly removed. Glands from each group were pooled and homogenized in 1.0 ml. of veronal buffer, $r/2 = 0.05$, pH 8.6, containing PTU, 7×10^{-4} M, in order to prevent further oxidation of iodide.[2] A measured aliquot of each homogenate

[2] Veronal buffer, as described herein, proved to be a convenient medium for homogenization of thyroid glands prior to tryptic hydrolysis. Stock solutions of this buffer, routinely employed for filter paper electrophoresis, are available in many laboratories, and provide stable conditions of pH, virtually optimal for the hydrolytic action of trypsin.

was counted in a well-type scintillation counter and its radioactivity compared with that of a suitable dilution of the administered dose of I^{131}. Percentile uptakes of radioiodine per rat thyroid were calculated.

Analysis of the Composition of Thyroidal I^{131}*:* To the remaining portions of each homogenate, approximately 4 mg. of powdered trypsin (Tryptar, Armour) were added. Hydrolysis was allowed to proceed at 38°C. for 24 hours. An additional 4 mg. of trypsin were added at the 15-hour interval. With this system of digestion, 90 per cent hydrolysis of thyroidal protein (as assessed by chromatographic migration of radioiodinated moieties) was routinely achieved.

Following digestion, 20 to 100 μl. of homogenate were applied to strips of Whatman No. 1 filter paper and were subjected to ascending chromatography in both butanol-acetic acid-water and butanol-dioxane-ammonia systems. Iodide, monoiodotyrosine (MIT), diiodotyrosine (DIT), thyroxine (T_4), and triiodothyronine (T_3) were employed as carriers. Subsequently, these were stained with palladium chloride, in the case of iodide, and diazotized sulfanilic acid, in the case of the iodinated amino acids. Developed chromatograms were analyzed with an automatically-recording strip scanner. Quantitative assessment of the relative proportion of the various radioiodinated compounds was achieved by means of a recording electronic integrator, developed in this laboratory. In this apparatus, each peak of the chromatogram produces a vertical deflection of the integrator, the height of which is directly proportional to the radioactivity of the peak. As the successive peaks are summated, the total height of the integral is proportional to the total radioactivity on the strip. Thus, the percentile contribution of an individual compound to the total radioactivity of a group of compounds can readily be calculated as the ratio of the height of the individual integral to the summated heights of the integrals of the group.

In the present experiments, the percentage of administered I^{131} incorporated into organic moieties (I^{131} organic-uptake) was calculated as follows:

$$\frac{I^{131}\text{ organic-uptake}}{\text{thyroid gland}} = \frac{\text{total } I^{131}\text{ uptake}}{\text{thyroid gland}} \times \%\text{ glandular activity organically-bound.}$$

The percentage distribution of I^{131} among individual iodinated amino acids was determined from those amino acids which migrated freely during chromatography following hydrolysis. It was assumed that similar ratios obtained in the unhydrolyzed thyroprotein which remained at the origin during chromatography.

RESULTS

For all groups of animals studied, Table 1 lists the doses of antithyroid agents employed, total I^{131} uptake and I^{131} organic-uptake, as well as the distribution of radioiodine among the various iodinated amino acids studied.

Control Groups: Excellent agreement was noted among the data obtained in control groups from the several experiments performed. In nine groups of three or four rats, total thyroidal I^{131} uptake averaged $2.6 \pm 0.4\%$ (Mean $\pm$ S.D.) of the administered dose per rat. Organic uptake of I^{131} averaged $2.3 \pm 0.4\%$ per rat. MIT comprised $33 \pm 3.1\%$ of organically-bound I^{131}, DIT $53.0 \pm 3.4\%$, and T_4 $13.5 \pm 4.0\%$. T_3 was identified on less than half the chromatograms and, when present, accounted for 1 to 3% of the organically-bound radioactivity.

PTU Group: Administration of increasing doses of PTU resulted in a

progressive diminution in I^{131} organic-uptake, and a striking alteration in the relative proportions of the various radioiodinated amino acids. With decreasing percentile incorporation of I^{131} into organic moieties, there occurred a reduction in the relative proportions of T_4* and DIT*, together with an increase in the percentage of MIT* (Fig. 1).[3] Thus, with a 16-fold decrease in organic-uptake of radioiodine (0.14% of administered dose), no radioactivity was detectable among the thyronines, and the DIT*/MIT* ratio was reduced from a control value of 1.6 ± 0.2 to 0.5 (Fig. 2). Further reductions in I^{131} organic-uptake were associated with more pronounced

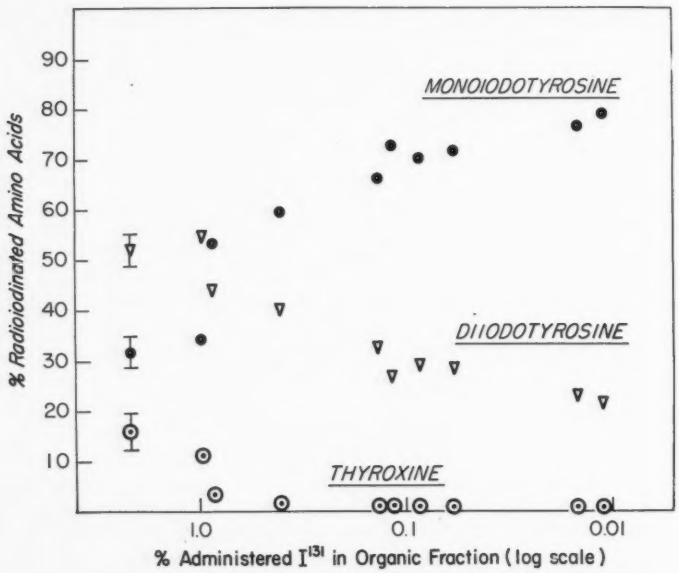

FIG. 1. The relationship between I^{131} organic-uptake and the percentage composition of radioiodinated amino acids in the thyroid glands of rats given graded doses of propylthiouracil. Control values ± S.D. are indicated by bracketed points.

decreases in the DIT*/MIT* ratio. As T_3 radioactivity was not uniformly detectable on chromatograms of control thyroid tissue, an accurate assessment of alterations in the amount of this compound, relative to the other radioiodinated amino acids, was not possible. However, in no instance could T_3 be detected when PTU had decreased the percentile radioiodination of T_4.

Perchlorate Group: Results in animals given perchlorate contrasted sharply with those obtained in animals given PTU (Figs. 2, 3), Over a range of I^{131} organic-uptake from 2.3% (control value) to 0.1% of the ad-

[3] The asterisk indicates I^{131}-labelled compound.

ministered dose per rat, the relative proportions of the individual radioiodinated amino acids were not appreciably altered. At an organic-uptake of only 0.1%, thyroidal T_4* content was similar to control values in the perchlorate group, but undetectable in the group given PTU. Similarly, the DIT*/MIT* ratio was unchanged from the control value of 1.6 in the perchlorate group, while reduced to a value of 0.4 in the animals given PTU. With extreme reduction in both total and organic-uptake of radioiodine, following large doses of perchlorate, a small decease in the DIT*/MIT* ratio occurred. At an organic-uptake of 0.006%, the ratio had de-

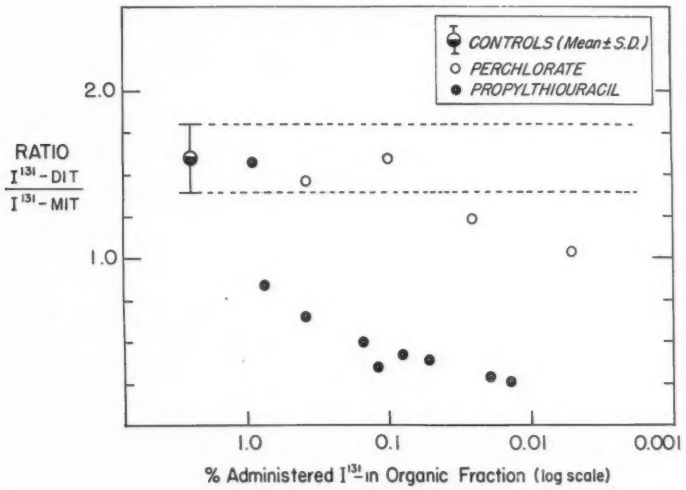

FIG. 2. The relationship between I^{131} organic-uptake and the I^{131}-DIT/I^{131}-MIT ratio in thyroid glands of rats given graded doses of perchlorate or propylthiouracil.

creased to 1.05. However, the relative proportion of T_4* in these animals did not differ appreciably from the control value.

TSH+PTU Group: In animals given no antithyroid drug, TSH induced a 2.4-fold increase in the I^{131} organic-uptake. However, the distribution of radioiodine among the various amino acids was similar to that found in the treatment-free controls. Increasing doses of PTU in animals given TSH resulted in successive decrements in I^{131} organic-uptake, associated with a progressive decrease in the relative proportions of T_4* and DIT*. Such decreases were evident in animals whose organic-uptakes were 160% of values found in treatment-free controls. In animals given sufficient PTU to reduce the organic accumulation of I^{131} by as little as 30% from values found in treatment-free controls, T_4* was absent, and the DIT*/MIT* ratio (0.8) was only half that found in the controls.

DISCUSSION

Observations in patients receiving propylthiouracil therapy for hyperthyroidism suggested that the therapeutic action of the thioureylenes might be more complex than had generally been supposed. Such patients not infrequently continue to accumulate in their thyroid glands a greater than normal proportion of administered I^{131}, despite a return to the eumetabolic state. This iodine is presumed to be organically bound, since it cannot be discharged by thiocyanate or perchlorate. Evidence has been presented which suggests that increased uptake of I^{131} is not conditioned

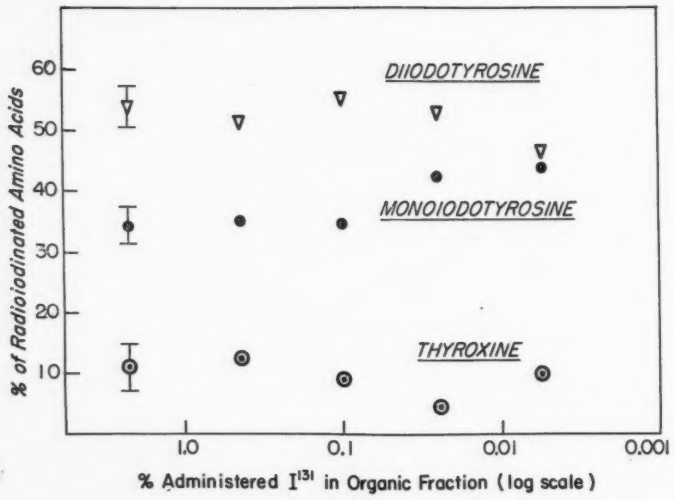

FIG. 3. The relationship between I^{131} organic-uptake and the percentage composition of radioiodinated amino acids in the thyroid glands of rats given graded doses of perchlorate. Control values ± S.D. are indicated by bracketed points.

by a deficiency of iodine, but indeed reflects thyroidal accumulation of excessive quantities of organified iodine. From this it was inferred that the antithyroid drug had favored the synthesis of compounds which either failed to reach the circulation, or which, in the periphery, had little calorigenic effect (4).

The present experiments lend this hypothesis substantial support. In rats, the acute administration of increasing doses of PTU, followed by tracer doses of I^{131}, was associated with a progressive reduction in the per cent of administered radioiodine incorporated into organic moieties (I^{131}-organic uptake). This effect was consonant with the classical concept of thioureylene action, *viz.*, inhibition of the initial oxidation and organification of thyroidal inorganic iodide. However, other effects were also evident. As the I^{131} organic-uptake decreased, an increasing percentage of

glandular radioiodine was found in the form of MIT, while the percentile contribution of DIT declined, and that of the thyronines became undetectable. These effects were noted as the total I^{131} uptake and the I^{131} organic-uptake were reduced to subnormal values. A closer analogy to the clinical situation was found in rats given TSH. Here, effects of PTU on the percentile composition of the individual radioiodinated amino acids were noted when uptake were above or within the normal range.

It might be anticipated that as the MIT*/DIT* ratio increased, and as long as formation of thyronines persisted, an increase should have occurred in the proportion of the less highly iodinated thyronines, relative to the proportion of T_4*. This was not observed under the present experimental conditions. On the contrary, under the influence of PTU, T_4* was the sole radioiodinated thyronine detected. Thus, a diminution in the relative proportion of T_4* reflected a decrease in the formation of all coupled products. It remains possible that a more prolonged period between injection of I^{131} and sacrifice of animals might have facilitated demonstration of an increase in the diiodothyronine*/T_4* or the T_3*/T_4* ratio. Nevertheless, it is clear that if the present results are to be extrapolated to the clinical situation, the important finding is the decrease in the total quantity of hormonally-active thyronines formed. Final proof of the putative sequence in patients receiving this drug, however, must await measurements of the thyroidal incorporation of stable iodine, together with demonstration of increased quantities of calorigenically-inactive products in their circulation or their thyroid glands.

It was of further interest to ascertain whether these were direct effects of PTU, or whether they might be secondary to an inhibition of initial organic iodinations. Alterations in the proportions of the iodinated amino acids have been found in the thyroid glands of rats maintained for long periods on diets deficient in iodine (7, 8). Propylthiouracil, by decreasing the quantity of oxidized iodine available for organic iodinations, might reduce the flux of stable iodine into organic moieties to an extent comparable to or in excess of that produced by dietary iodine deficiency. Alterations in iodination patterns might then result from this "intrathyroidal iodine deficiency." To assess this possibility, experiments were performed using perchlorate as a blocking agent. By selecting suitable doses of perchlorate, it was possible to produce decreases in I^{131} organic-uptake comparable to those produced by PTU. Although not measured, it seems safe to assume that appreciable differences did not exist between the specific radioactivities of extracellular iodide in the two treatment groups. Thus, by inhibiting the trapping of inorganic iodide, perchlorate should have produced effects comparable to those of PTU as regards a) decreased availability of oxidized iodine, b) decreased flux of stable iodine into organic moieties, and c) loss of glandular hormonal stores. In so doing, however, perchlorate had little or no effect on iodination patterns within the gland. The present experi-

ments therefore indicate that acute decreases in the quantity of iodine available for organic iodinations, as well as the resulting reduction in glandular hormonal stores, can not account for the effects of PTU on the nature of the iodinated amino acids formed. Experiments performed in animals given TSH, cited above, lend further weight to this conclusion.

Thus PTU appears to exert a specific inhibitory effect upon a) the diiodination of MIT and b) the coupling of MIT and DIT to form the several iodothyronines. On the basis of data reported in abstract form, Slingerland has concluded that PTU has no effect on the coupling reaction (9). However, it would appear from the present data that the coupling reaction may be more sensitive to the action of PTU than is the formation of DIT, since at higher doses of antithyroid agent, thyronines became undetectable, while formation of DIT* continued. Little is known of the chemical mechanism whereby coupling occurs in living tissue, but it appears to be a remarkably labile system. Surviving thyroid slices apparently have little capacity to synthesize iodothyronines (10). In purely chemical systems, coupling of DIT (11) and its derivatives can be effected under oxidative conditions (12, 13). The observation that yields of iodothyronines are improved when DIT is bound in peptide linkage (14) supports the suggestion that the formation of the thyronines *in vivo* occurs within the matrix of the thyroglobulin molecule (15). If this be true, then the inhibitory action of PTU may arise from an effect of this agent upon the synthesis of thyroidal protein or upon the molecular rearrangements incident to the coupling process. Of interest in this regard is a report that in dogs chronically treated with PTU, thyroglobulin displays abnormal solubility characteristics in salt solutions of high ionic strength (16).

During *in vitro* iodination of tyrosine, the relative proportions of DIT and MIT depend on the quantity of iodine present (17). In cellular and subcellular systems of thyroidal tissue this seems clearly not the case. Monoiodotyrosine has been reported as the sole form of organically-bound iodine in the $8\frac{1}{2}$ day old chick embryo (18). Similarly, thyroid slices stored at 4° C. (19) and homogenates of thyroid tissue (10) are capable of synthesizing MIT, but not DIT, despite large increments in the availability of iodide. In addition, hydrolysates of certain non-toxic nodular goiters (20) and thyroidal carcinomas (21) may reveal a very high percentage of iodinated amino acids as MIT. The present data provide further evidence of the dissociation of the mono- and diiodination reactions *in vivo*.

As might be anticipated from the paucity of knowledge concerning the precise chemical mediation of hormonal biosynthesis, little is known of the mechanism of action of the antithyroid agents. Dependence of iodinations upon the action of an oxidative catalyst has been generally accepted, and the peroxidase system has been most frequently implicated (5). Since the thiouracils quickly reduce molecular iodine, prevent the iodination of casein (22), and inhibit the coupling of DIT to yield T_4 *in vitro* (13), it is

tempting to ascribe all of the inhibitory actions of PTU to its reductive capacity. In view of the present data, this hypothesis would necessitate the inference that a progressively greater oxidative potential is required for successive stages in hormonal biosynthesis. At present, techniques for directly assessing this possibility are not readily available.

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RELATIONSHIP BETWEEN THYROID FUNCTION AND CARDIOTOXIC PROPERTIES OF ISOPROTERENOL

CLIFFORD I. CHAPPEL, GEORGE RONA AND ROGER GAUDRY

Ayerst, McKenna and Harrison Limited, Montreal, Quebec, Canada

ABSTRACT

The relationship between thyroid function and response to a synthetic adrenergic agent, isoproterenol, has been studied in the rat. Hypothyroidism after thyroidectomy or treatment with propylthiouracil for one or three weeks resulted in milder symptoms, reduced heart weights and less severe heart lesions after isoproterenol treatment than was seen in the controls. Thyroxine treatment on the other hand increased the mortality and heart weight after isoproterenol but had less marked effects on the myocardial necrosis. The myocardial infarct-like necrosis produced by isoproterenol is believed to be due to local ischemia resulting from a relative hypoxia in the myocardial fibres stimulated by this drug. The relationship seen between thyroid function and isoproterenol sensitivity supports this concept.

THE investigation of factors affecting myocardial necrosis and healing in experimental animals has been handicapped by the lack of a simple method for producing uniform lesions. During the course of comparative toxicological studies with sympathomimetic amines we noted that 1-(3,4-dihydroxyphenyl)-2-isopropyl amino ethanol hydrochloride (isoproterenol) produced infarct-like myocardial necrosis in the rat (1). By injecting varying doses of this material subcutaneously on two consecutive days, myocardial lesions of predictable severity could be produced (2), with a direct relationship between the weight of the animals and the severity of the lesions (3). Isoproterenol was found to be more potent in producing myocardial infarcts than epinephrine or arterenol (4).

It has been known for many years that important functional interrelationships exist between the thyroid gland and the hormones of the adrenal medulla. It has been shown in different species that a decreased sensitivity to epinephrine was present after thyroidectomy (5, 6, 7, 8) while the administration of thyroid hormone markedly increased adrenalin-sensitivity (9, 10, 11, 12). On the basis of the increased myocardial metabolism (13, 14) in hyperthyroidism and the reduced sensitivity to epinephrine in the hypothyroid state (15, 16) total thyroidectomy (17, 18), antithyroid drug treatment (19, 20), and radioactive iodine (21, 22) have been recommended as therapeutic measures in angina pectoris.

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This study was performed to determine whether similar relationships might be demonstrated between thyroid function and the synthetic adrenergic agent isoproterenol, and whether alterations in isoproterenol sensitivity would be reflected in the severity of myocardial lesions produced by this drug in the rat.

EXPERIMENTAL

A total of 240 male Wistar rats weighing an average of 200 ± 5 gm. were used in this study. They were housed under controlled conditions at a temperature of $75 \pm 2°F$ and a relative humidity of $45 \pm 5\%$. They were fed a commercial rat food (M. Fortin, Hemmingford, Que.). Isoproterenol was given subcutaneously as the hydrochloride of the racemic mixture on two consecutive days at doses of 85, 5.25, and 0.33 mg./kg. Each of the three dose levels of isoproterenol was administered to a group of 10 rats after the following pretreatments. 1. Thyroidectomy one week before isoproterenol treatment. 2. Propylthiouracil (PTU) 20 mg./kg./day orally for 1 and 3 weeks before the isoproterenol. 3. Thyroxine administered in the DL, form at 100 μg. and 250 μg./rat/day subcutaneously starting 1 week prior to treatment with isoproterenol. PTU and thyroxine were administered in aqueous solution. A control group of rats was treated with 1 ml. of distilled water orally each day for 1 week before giving each dose level of isoproterenol and groups of rats were also studied after thyroidectomy, PTU and thyroxine treatment without isoproterenol.

The symptoms and mortality in each group were recorded during the experiments. Forty-eight hours after the first isoproterenol treatment or at the corresponding time in the control group the rats were killed and autopsied. The tissues were removed and weighed, and gross lesions of the heart were graded. After fixation in Bouin's fluid frontal sections of the heart, which included both atria, ventricles and interventricular septum, were embedded in paraffin. Sections cut at 5 μ were stained with haematoxylin-eosin, Cason's trichrome (23) and P.A.S. (24). Sections were also made from the brain, pituitary, thymus, thyroid (where it was present), lung, liver, kidney and adrenal.

The grading system which we have described in detail elsewhere (2) may be summarized as follows:

Gross Lesion—

Grade 0: No lesions.
Grade 1: Mottling of the apex and distal parts of the left ventricle caused by intermingled pale and dark red streaks.
Grade 2: Well demarcated necrotic areas limited to the apex.
Grade 3: Large infarct-like necrosis involving at least one-third of the left ventricle and extending to adjacent areas of the interventricular septum and right ventricle.
Grade 4: Large infarct-like necrosis involving more than half of the left ventricle, the interventricular septum and extending to the distal portion of the right ventricle.

Microscopic Lesion—

Grade 0: No lesions.
Grade 1: Focal lesions of the subendocardial portion of the apex and/or the papillary muscle composed of fibroblastic swelling or proliferation and accumulation of histiocytes.

Grade 2: Focal lesions extending over wider areas of the left ventricle with right ventricular involvement, lesions included edema, mottled staining, fragmentation and segmentation of muscle fibres.

Grade 3: Confluent lesions of the apex and papillary muscles with focal lesions involving other areas of the ventricles and atria. The lesions included vacuolar degeneration, granular disintegration, and hyaline necrosis of the muscle fibres, marked capillary dilatation and hemorrhages, pronounced mononuclear cell and leukocytic infiltration and extensive edema, occasionally with a mucoid component.

Grade 4: Histologically the lesions were similar in character to those of Grade 3 but extending throughout the heart. The lesion was usually a massive infarct-like necrosis with occasional acute aneurysms or mural thrombi.

RESULTS

Symptoms and Mortality

The control rats which received distilled water and were then treated with either 85 or 5.25 mg./kg. of isoproterenol showed characteristic symptoms. Within 20 minutes of treatment the animals assumed unusual postures, some lay on their side or back with the head extended, others supported themselves on the side of the cage and extended their head and front legs. In either position they breathed with their mouth open, respirations being dyspnoeic, irregular and abdominal. Many of these animals had marked edema of the head, neck, chest and front legs. No symptoms were observed in the group treated with 0.33 mg./kg. isoproterenol. As shown in Table 1 only one death occurred in the animals receiving isoproterenol alone. This animal was from the group which received 85 mg./kg.

A marked difference was evident in the symptoms shown by the animals which were hypothyroid after thyroidectomy or PTU treatment. The hypothyroid rats treated with 0.33 or 5.25 mg./kg. of isoproterenol showed no symptoms while the group treated at 85 mg./kg. showed a barely discernible dyspnoea. No deaths were seen in any of the animals thyroidectomized or pretreated with PTU.

There was little difference in the symptoms of the groups treated with either 100 or 250 μg. of thyroxine. At either 85, 5.25 or 0.33 mg./kg. of isoproterenol the symptoms shown by these animals were more severe than those shown by the controls. Within a few minutes of isoproterenol treatment the animals were prostrated and in a state of collapse. The symptoms shown by the hyperthyroid animals lasted longer than those in the controls and as shown by the mortality figures in Table 1 many of these animals failed to recover from these immediate effects of isoproterenol treatment.

Tissue Weights

In earlier studies we noted that isoproterenol treatment caused an increase in the weight of the heart in the rat and that an inverse relationship

existed between the size of the animal and the degree of cardiac hypertrophy (3). The animals used in this study had a uniform body weight and consistent changes were seen in the heart weights of the different groups. The rats treated with isoproterenol and distilled water had a significant cardiac hypertrophy (Table 1) which was related to the dose used. Thyroxine treatment alone caused a slight though insignificant increase in heart weight, and when thyroxine treated animals were given isoproterenol an additive effect of the two treatments was seen on the heart weight. This aggravation of the effect of isoproterenol by thyroxine is evident in the hyperthyroid groups treated at 5.25 or 85 mg./kg. of isoproterenol.

A significant depressant effect on heart weight was observed in the thy-

TABLE 1. EFFECT OF THYROID FUNCTION ON HEART LESIONS PRODUCED BY ISOPROTERENOL

Group	Isoproterenol 85 mg./kg.						Isoproterenol 5.25 mg./kg.					
	Thyro X	PTU		Thyroxine		Dist. water	Thyro X	PTU		Thyroxine		Dist. water
		1 week	3 weeks	100 μg.	250 μg.			1 week	3 weeks	100 μg.	250 μg.	
Mortality	0	0	0	3	4	1	0	0	0	2	4	0
Heart Weight mg./100 gm. B.W.	408± 18	506± 10	496± 18	623± 16	598± 19	562± 13	373± 4	460± 12	418± 8	631± 17	632± 37	517± 6
Average Gross Lesion	0.9± 0.2	2.6± 0.2	1.2± 0.2	3.5± 0.2	3.2± 0.2	3.3± 0.2	0.5± 0.1	1.4± 0.2	0.9± 0.2	2.0± 0.2	2.4± 0.3	2.2± 0.2
Average Microscopic Lesion	1.3± 0.2	2.9± 0.2	2.0± 0.1	3.9± 0.1	3.8± 0.1	3.8± 0.1	0.9± 0.2	2.5± 0.2	1.5± 0.2	3.4± 0.2	3.9± 0.1	3.0± 0.2
	Isoproterenol 0.33 mg./kg.						Controls					
Mortality	0	0	0	2	1	0	0	0	0	0	0	0
Heart Weight mg./100 gm. B.W.	363± 24	369± 8	397± 14	526± 15	558± 13	509± 26	290± 5	350± 10	318± 14	454± 15	447± 17	414± 18
Average Gross Lesion	0	0.7± 0.1	0.3± 0.1	1.9± 0.3	2.1± 0.3	1.5± 0.2	0	0	0	0	0	0
Average Microscopic Lesion	0.6± 0.1	1.0± 0.2	0.8± 0.1	2.5± 0.3	2.7± 0.3	2.3± 0.2	0	0	0	0	0	0

roidectomized rats or those treated with PTU without isoproterenol. When these hypothyroid rats were treated with isoproterenol at 0.33, 5.25 or 85 mg./kg. a consistent depressant effect on the isoproterenol induced cardiac hypertrophy was observed.

There was a great variability in the weights of other tissues in these animals. This variation was primarily due to different degrees of passive congestion in the organs of the animals within each group. However the alterations in thyroid function also had effects on tissue weights.

Cardiac Lesions

The mean of the gross and histological grading of the heart lesions are presented in Table 1. Isoproterenol treatment alone caused marked heart lesions (Fig. 1) the severity of which was directly related to the dose. Thyroxine treatment alone caused a mild interstitial edema and focal

FIG. 1. Massive infarct-like myocardial necrosis (Grade 4) produced in the rat by injecting 85 mg./kg. of isoproterenol on two successive days. Examples are epicardial surface and frontal sections of the same heart.

swelling of fibroblasts; these changes were observed in two animals treated at 100 μg. and six treated at 250 μg. These lesions were minimal and not of sufficient severity to grade as 1. In general, hyperthyroidism after thyroxine treatment resulted in an aggravation of the cardiac lesions of isoproterenol. This increase in severity was most evident at the lowest level of isoproterenol treatment. At either 5.25 or 85 mg./kg. the lesions produced by isoproterenol treatment alone were quite severe and the aggravation of these lesions was not so apparent in the grading. Histologically, however, the lesions in the hyperthyroid animals given isoproterenol were more extensive, proliferation of fibroblasts was more intense with large numbers of Anitschkow cells and mitotic figures present. The rats which were hypothyroid following thyroidectomy or PTU treatment showed no evidence of cardiac changes. When these animals were treated with isoproterenol a marked protective effect was observed. The reduction in severity of the myocardial lesions was greatest in the thyroidectomized groups, somewhat less in the groups treated for three weeks with PTU and least in the group treated with PTU for one week. This relationship between treatment and degree of protection was seen in the animals treated with 0.33, 5.25 or 85 mg./kg. of isoproterenol.

Other Organs

The animals which were thyroidectomized or treated with thyroxine or PTU showed the changes in the thyroid, adrenal and pituitary which are well known (25). The thyroxine treated rats also showed changes in the liver and kidney similar to those which have been described in hyperthyroid animals (26, 27, 28).

DISCUSSION

In earlier studies we reported that subcutaneous administration of isoproterenol to the rat produced infarct-like myocardial necrosis of predictable severity which was clearly related to the dose used (1, 2). The lesions after treatment with this drug were more severe than those produced by l-epinephrine (4) and occurred at doses below those which caused mortality. Thus isoproterenol-induced cardiac necrosis appeared to be a suitable model for the study of factors which affect myocardial lesions produced by adrenergic drugs. It is evident from the findings reported herein that a direct relationship exists between thyroid function and the effects of isoproterenol on the heart. This relationship is particularly clear in the animals which were thyroidectomized or treated with PTU. At every level of isoproterenol these animals had lower heart weights and less severe heart lesions than the respective controls. Furthermore the symptoms shown by these animals were quite mild. Thus in every aspect which was studied hypothyroidism was seen to exert a protective action against the effects of isoproterenol. This situation is somewhat more complex in the animals which were treated with thyroxine. The symptoms shown by these rats were more severe than in the animals which were euthyroid before giving isoproterenol; they also had a higher mortality rate and a greater heart size. However the aggravation of the effects of isoproterenol by hyperthyroidism are not so clear when the mean grades of the gross or microscopic myocardial lesions are compared. At the highest dose of isoproterenol, 85 mg./kg., the lesions in the controls were almost maximal so the failure of thyroxine treated rats to develop more severe lesions is not surprising. However we are unable to account for the failure of the hyperthyroid rats treated with lower doses of isoproterenol to develop lesions of obviously greater severity than the controls, particularly since thyroid overdosage alone is known to produce cardiac hypertrophy (29, 30) and focal myocarditis (31, 32) and aggravate myocardial lesions produced by other agents (31, 33).

We have postulated earlier (3, 4) that the cardiotoxicity of isoproterenol is related to the cardiovascular effects of the drug rather than to a direct toxic effect exerted on the heart. The basis of the lesions is believed to be a hypoxia resulting from an increase demand for oxygen created by cardiac stimulation which exceeds the supply available from the coronary circula-

tion. The results of this study support this contention. Hyperthyroidism which increases the oxygen demands of the myocardium markedly augments the sensitivity of the rat heart to isoproterenol resulting in an increased mortality, cardiac enlargement and a slight increase in the myocardial necrosis. The lowering of the myocardial oxygen consumption in hypothyroidism on the other hand dramatically reduces the degree of myocardial enlargement and necrosis produced by isoproterenol.

It would appear therefore that the relationship between thyroid function and isoproterenol sensitivity is similar to that which exists between the thyroid state and sensivity to epinephrine. Earlier workers have demonstrated adrenergic-thyroid interrelationships in terms of pharmacologic responses to epinephrine (34, 35). In this experiment the same interrelationships have been shown to exist when morphological responses to a synthetic adrenergic drug are studied.

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STUDIES ON THE MECHANISM OF HYPOTHALAMIC CONTROL OF THYROTROPIN SECRETION: EFFECT OF INTRAHYPOTHALAMIC THYROXINE INJECTION ON THYROID HYPERTROPHY INDUCED BY PROPYLTHIOURACIL IN THE RAT[1]

TAKASHI YAMADA

Division of Endocrinology, Department of Medicine, University of Oregon Medical School, Portland, Oregon

ABSTRACT

A minute amount of thyroxine was injected into the anterior hypothalamus of rats chronically treated with propylthiouracil. The subsequent development of goiter was studied in relation to the hypothesis proposed in a previous paper that the "thyrotropin center" of the anterior hypothalamus is sensitive to alterations in the concentration of circulating thyroid hormone and thus can secondarily control thyrotropin secretion by the pituitary. The development of goiter was markedly inhibited by the injection of systemically ineffective amounts of thyroxine into the anterior hypothalamus but not by saline injections into the same area or by thyroxine injections into other areas of the hypothalamus. Adrenal and testicular weight were not affected by such procedures. It is suggested that thyroxine injection into the anterior hypothalamus specifically affects thyrotropin secretion by the pituitary.

THERE is strong evidence from a number of investigations that the hypothalamus participates in regulating the secretion of pituitary trophic hormones by means of some unknown substance which passes through the hypophyseal portal vessels from the median eminence of the hypothalamus to the anterior pituitary (1, 2). In considering the classical "feed-back theory" (3), it is reasonable to expect that such hypothalamic control might be explained by assuming that the circulating target organ hormones can inhibit the activity of appropriate hypothalamic centers and thus induce a secondary suppression in the secretion of trophic hormones from the pituitary. Recent studies by von Euler and Holmgren (4, 5) have suggested that thyroid hormones can inhibit thyrotropin secretion only through a direct effect on the anterior pituitary and that the hypothalamus is not responsive to altered thyroid hormone levels. Since hypothalamic lesions can significantly depress thyroidal activity (6, 8, 7), however, it seems inappropriate to exclude the hypothalamus as a reactive site without further study.

A previous publication from this laboratory (9) has demonstrated that

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thyroxine injected into either the anterior pituitary or the anterior hypothalamus can inhibit thyroidal release of radioiodine. This suggests that an increased level of circulating thyroid hormone might act at both the pituitary and hypothalamic levels to suppress TSH secretion.

In the present study, another parameter of altered thyrotropin secretion was investigated. A systemically ineffective amount of thyroxine was injected into the anterior hypothalamus of animals chronically treated with propylthiouracil to ascertain whether inhibition of thyroid hypertrophy would be produced.

MATERIALS AND METHODS

Including a preliminary experiment 65 adult, male Holtzman rats were employed. The animals were divided into 6 groups: I. Hypothalamic thyroxine injection+propylthiouracil (PTU); II. Hypothalamic saline injection+PTU; III. PTU Control; IV. Untreated Control; V and VI. Graded doses of systemically injected thyroxine+PTU.

The following experimental procedures were employed in each of the respective groups for 9 days: (I) 0.25 μg. of L-thyroxine (T_4) dissolved in 0.02 ml. physiological saline was injected into the hypothalamus twice daily under light ether anesthesia (total daily dose = 0.2 μg. T_4/100 gm. body weight); (II) 0.02 ml. physiological saline was injected twice daily in the same manner; (V and VI) Graded doses of T_4 were dissolved in 0.25 ml. physiological saline and administered intraperitoneally twice daily (total daily dose = 0.2, 0.5 and 1.0 μg. T_4/100 gm. body weight). PTU (30 mg. in 1 ml. of 10% gum acacia) was injected subcutaneously once daily at rotated sites in groups I, II, and III, while it was fed in the diet at a concentration of 0.15% in Groups V and VI. All groups were fed Purina laboratory checkers except Groups V and VI which received a Remington low iodine diet. The animals were killed on the 10th day of the experiment. Thyroid, adrenals, and testes were dissected and weighed. The hypothalamus and pituitary were also removed and fixed in Bouin's fluid. Ten μ serial sections were stained with hematoxylin and eosin.

Intrahypothalamic injections were made through an implanted #27 hypodermic needle as previously described (9).

RESULTS

I. *Preliminary experiment*

In an exploratory study, the right lobe of the thyroid gland was removed in 11 animals. All were injected with 30 mg. of PTU daily as described above and 7 were given intrahypothalamic thyroxine as described for Group I under Methods. At autopsy the left lobe was removed and weighed. The left lobe/right lobe ratio was taken as an index of thyroid hypertrophy. Unfortunately adequate dissection of the left lobe was difficult due to adhesions from prior surgery, so that the results were difficult to interpret and are not recorded in detail here. Nevertheless, it should be mentioned that the ratio in the group in which thyroxine was injected into the anterior hypothalamus was much smaller than that of the control group while the ratio in animals which received thyroxine injections into the posterior hypothalamus was similar to that of the control group. Because of this and similar results in previous studies (9), attention in the following experiments was concentrated on the anterior hypothalamus.

TABLE 1. SUMMARY OF THE EFFECTS OF SALINE AND THYROXINE INJECTIONS INTO THE HYPOTHALAMUS ON THYROID, ADRENALS AND TESTES IN RATS CHRONICALLY ADMINISTERED PROPYLTHIOURACIL[1]

Experiment and Group	No. of Animals	Initial Body Wt. (gm.)	Final Body Wt. (gm.)	Thyroid		Adrenals		Testes	
				mg.	mg./100 gm.	mg.	mg./100 gm.	gm.	gm./100 gm.
I. T_4	8	240±2	199± 6	19.29±2.29	8.1±0.9	55.8±2.7	23.3±1.2	2.97±0.05	1.23±0.03
II. Saline	4	254±7	205±11	33.29±3.12	13.2±1.6	62.2±1.4	24.6±0.7	2.96±0.15	1.16±0.08
III. PTU Control	5	248±5	254± 8	34.05±2.64	13.7±1.2	63.9±1.5	25.8±0.5	3.23±0.05	1.30±0.01
IV. Untreated Control	4	256±3	272± 4	18.02±2.31	7.1±0.9	40.0±1.5	15.7±0.6	3.03±0.08	1.19±0.04

[1] Results expressed as mean ± standard error of mean. Groups I, II, and III were injected with 30 mg. PTU daily for 9 days and autopsied on the 10 day. Organ/body weight ratios are calculated on the basis of initial body weight due to the irregular loss of weight in some groups.

II. *Effect of hypothalamic thyroxine injection on the weight of thyroid, adrenals, and testes.*

The effect of intrahypothalamic thyroxine injection on the weights of certain endocrine glands is shown in Table 1. It is evident that the saline injections into the hypothalamus did not inhibit thyroid hypertrophy produced by PTU. In contrast, goitrogenesis was completely prevented in 7 of the 8 animals which received T_4 injections in the anterior hypothalamus.

An increase in adrenal weight was observed in all treated groups. The weight of the testes, on the other hand, was quite uniform in all groups in spite of the loss of body weight in the animals receiving intrahypothalamic injections.

III. *The effect of systemic injections of thyroxine on goitrogenesis induced by PTU.*

No inhibition of goiter formation was induced by the systemic injection of the same amount of T_4 that would prevent thyroid hypertrophy when injected into the anterior hypothalamus (Table 2). Doses of 0.2 or 0.5 μg. T_4/100 gm./day produced slightly larger goiters than the administration of PTU alone without added T_4, although the difference was not statisti-

TABLE 2. SUMMARY OF THE RESULTS OF SYSTEMIC THYROXINE INJECTION ON GOITER DEVELOPMENT[1]

Experiment and Group	T_4 (μg.) Daily	PTU	No. of Animals	Initial Body Wt. (gm.)	Final Body Wt. (gm.)	Thyroid	
						mg.	mg./100 gm.
V.							
1	—	—	4	144±3	201±2	10.47±0.52	5.2±0.3
2	—	+	4	129±3	155±6	29.98±1.59	19.4±0.9
3	0.2	+	4	156±3	161±6	33.58±2.17	21.0±1.3
4	0.5	+	4	138±3	149±3	30.49±3.92	20.4±2.4
5	1.0	+	4	165±3	164±3	30.06±4.45	18.4±2.6
VI.							
1	—	+	6	106±3	124±6	24.07±2.46	19.3±1.2
2	0.2	+	7	105±1	128±3	33.52±1.43	26.3±0.9

[1] Results expressed as mean ± standard error of mean. PTU was fed at 0.15% concentration in the diet for 9 days and the animals were autopsied on the 10th day. One half the daily dose of thyroxine was administered intraperitoneally every 12 hours.

cally significant. Only when the dose reached 1 μg. of T_4 daily did some decrease in thyroid size appear. The experiment was repeated with a 0.2 μg. dose. This time a significantly larger thyroid size was obtained with PTU plus T_4 than with PTU alone (Table 2).

IV. *Histological observations*

The anatomical location of the hypothalamic needles is shown in Figures 1 and 2. The tips of the needles were situated in the "thyrotropin area," which lies in the midline between the paraventricular nucleus and the median eminence, in both the thyroxine and saline control groups. In the

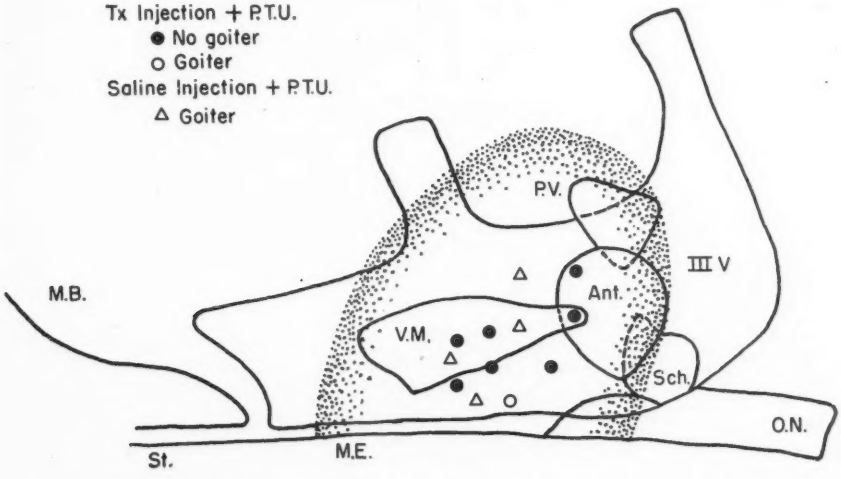
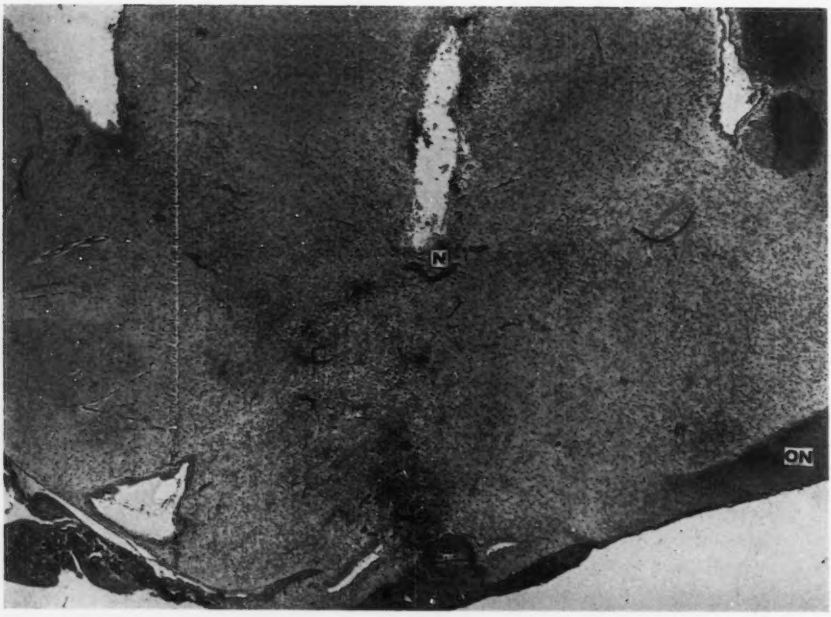


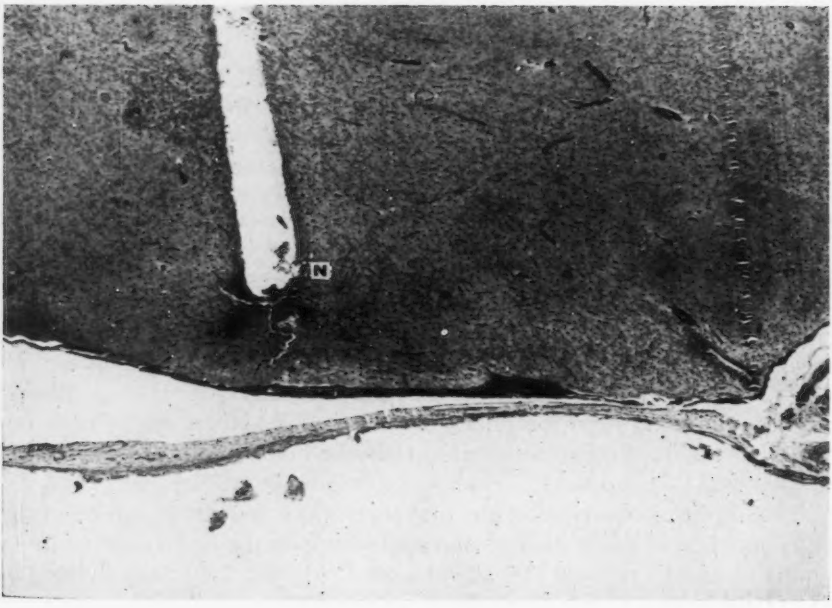
FIG. 1. Diagram of the position of the inserted needles in the hypothalamus. The thyrotropin area is delineated by stippling. P.V. = paraventricular nucleus, III V. = third ventricle, Ant. = anterior hypothalamic area, Sch. = Suprachiasmatic nucleus, V.M. = ventromedian nucleus, M.E. = median eminence, M.B. = mamillary body, St. = pituitary stalk.

animal in which no decrease in thyroid size was produced by intrahypothalamic thyroxine injection, the needle was also in the "thyrotropin area" but close to the inferior border of the hypothalamus (Fig. 1) so that some leakage of the injected material from the hypothalamus might have occurred. A slight cytological reaction could be noticed around some of the needles, but there appeared to be negligible damage or infection.

An interesting observation was that there was a marked dilatation of the capillary tufts of the hypophyseal portal system in the median eminence in animals in which thyroid hypertrophy occurred (Fig. 3A). Such changes in the portal system were absent or only minimally present in animals without thyroid hypertrophy (Fig. 3B).



(a)

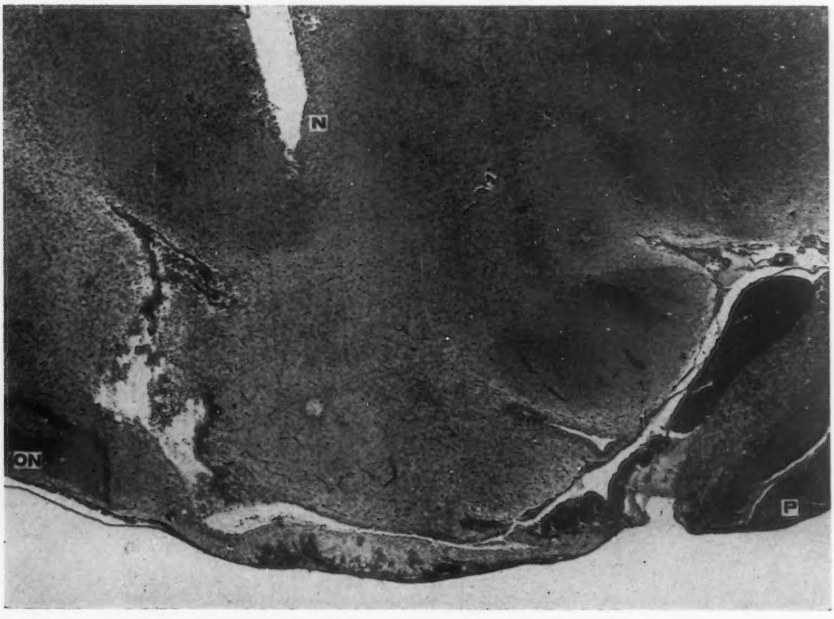


(b)

DISCUSSION

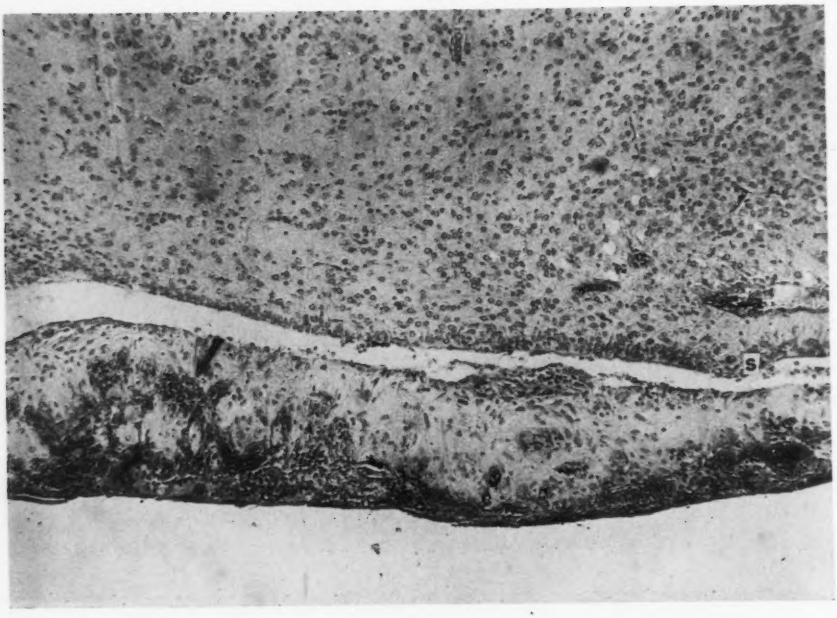
Flerkó and Szentágothai (10) have reported an inhibition of gonadrotropin secretion by the transplantation of tiny bits of ovary into the hypothalamus of the rat. They suggest that the functional state of the nerve cells located in the paraventricular nucleus is modulated by the blood level of estrogen and can thus regulate the secretion of gonadotropin by the pituitary. I have attempted to inhibit compensatory hypertrophy of the remaining thyroid gland after three quarter thyroidectomy by transplanting similar bits of thyroid into the hypothalamus (11). It was difficult to implant the tissue exactly in the intended location in the hypothalamus and also difficult, because of adhesions from prior surgery, precisely to dissect at autopsy the thyroid tissue left *in situ*. In the successful cases, however, the thyroid transplants produced an apparent inhibition of thyroid hypertrophy.

The present experiments clearly demonstrate that systemically ineffective amounts of thyroxine injected into the anterior hypothalamus will inhibit goiter formation produced by propylthiouracil. This confirms and extends the previous study in which intrahypothalamic thyroxine injec-

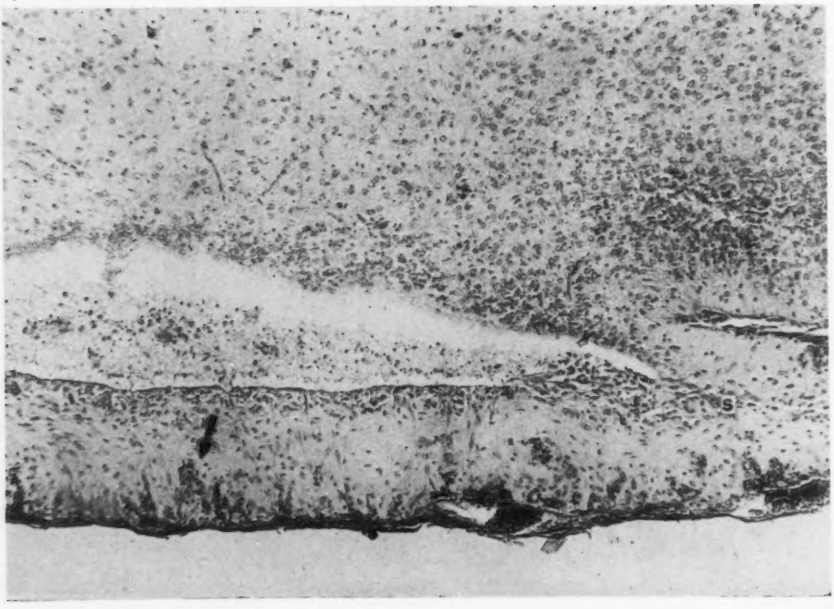


(c)

FIG. 2. Needles inserted into the anterior hypothalamus of rats: (a) and (b) photographs of sagittal sections of the hypothalami of thyroxine injected animals. (c) Sagittal section of the hypothalamus of a saline-injected animal. N = needle track, O.N. = optic nerve, P = pituitary.



(a)



(b)

tions markedly decreased the rate of release of radioactive iodine from the thyroid gland of the rat (9).

The different results obtained by von Euler and Holmgren (4) and by our laboratory (9) cannot be definitely explained at present. However, we injected a relatively large volume of fluid into the hypothalamus compared to the technique used by von Euler. Also, the site of injection was somewhat different since their hypothalamic injections were concentrated near the median eminence. Experiments from various laboratories have indicated that a reasonably large area of the hypothalamus must be destroyed before decreased secretion of thyrotropin is produced (1). Therefore it is possible that in order to produce a decrease in TSH secretion a greater area of the hypothalamus must be exposed to increased thyroxine concentration than was done in von Euler's experiments.

The enhancement of the goitrogenic action of propylthiouracil by small doses of thyroxine observed in this investigation is of considerable interest though further study is needed for confirmation. Similar results have been reported by Sellers *et al.* (12, 13) and Teir *et al.* (14), although the dose of thyroxine or desiccated thyroid as well as the duration of their experiments was different from those reported here. It is possible that a small dose of thyroxine may enhance the ability of the pituitary to secrete thyrotropin due to a nonspecific general increase in metabolic processes. Such an interpretation is supported by the fact that the pituitary of some patients with myxedema can secrete more thyrotropin immediately after therapy with thyroid hormone is begun than during the pretreatment period (15).

Thyroxine injection into the hypothalamus apparently specifically affects thyrotropin secretion by the pituitary without interfering with the other trophic hormones since the weights of both the adrenals and testes of the thyroxine injected animals were not significantly different from those of the saline-injected controls.

The present experiments did not indicate whether the inhibitory effect of intrahypothalamic thyroxine injections was produced by direct action on neural elements in the hypothalamus or by diffusion of the injected thyroxine through the hypophyseal portal vessels to the anterior pituitary. However, other studies have shown that the dose necessary to produce inhibition is roughly equivalent with thyroxine injected into either the hypothalamus or pituitary (16). Also radiothyroxine diffuses rapidly from the site of injection in the hypothalamus but is not significantly concentrated

FIG. 3. Histological findings in the stalk and the median eminence in 10 μ sections stained with H & E. Primary plexus of the hypophyseal portal system in (a) an animal that became goitrous and (b) an animal that did not. Note dilatation of the primary plexus of the portal system in (a) compared to (b). Arrow = primary plexus of portal system; S = pituitary stalk.

in the pituitary gland (16). These facts strongly suggest the existence of both a hypothalamic and an anterior pituitary receptor responsive to alterations in the level of circulating thyroxine.

Acknowledgment

I am deeply indebted to Dr. Monte A. Greer for helpful suggestions and advice during the course of this investigation.

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HYPERPLASIA OF THE ADRENAL CORTICAL TISSUE IN PACIFIC SALMON (GENUS *ONCORHYNCHUS*) AND RAINBOW TROUT (*SALMO GAIRDNERII*) ACCOMPANYING SEXUAL MATURATION AND SPAWNING

O. H. ROBERTSON AND BERNARD C. WEXLER[1,2]

Department of Biological Sciences, Stanford University, Stanford, California, and The May Institute of the Jewish Hospital and Medical Center and the Department of Pathology, University of Cincinnati, College of Medicine, Cincinnati, Ohio

ABSTRACT

A comparative study was made of the histological changes occurring in the adrenal cortical tissue of the Pacific salmon, the sea-run steelhead trout, and the non-migratory rainbow trout at various stages in their life cycles with the purpose of ascertaining whether there exists a relationship between degree of adrenal hyperplasia at spawning and survival thereafter. These three fishes show varying post-spawning outcomes from death of all the salmon to partial mortality in steelhead to usual survival of the rainbow trout.

The adrenal cortical tissue, found in the cephalic end of the kidney, consists in the sexually immature fish of small isolated groups of characteristic cells which are usually associated with branches of the cardinal vein. With the onset of gonadal development the adrenal cells begin to increase in number and by the time sexual maturity is reached in the salmon and steelhead, marked hyperplasia is present. The cells of this hyperplastic tissue are larger than those in the immature fish and are uniform in size and appearance. In the spawning fish degeneration of the adrenal cells is evident—this is more marked in the salmon than in the steelhead trout—many of which show no degenerative change. In contrast to the salmon and steelhead, hyperplasia of the adrenal tissue in the non-migratory rainbow trout is very much less pronounced. The various factors which might operate to induce adrenal hyperplasia are discussed.

IN A previous communication (1) we described briefly the occurrence of adrenal cortical tissue hyperplasia in the spawning Pacific salmon. Since it seemed possible that hyperactivity of the adrenal gland might well play a major role in bringing about the marked deterioration and death of this salmon which occurs regularly after its first spawning, we have amplified the previous observations and extended the study to include other closely related salmonids, namely migratory steelhead and non-migratory rainbow

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trout. Steelhead trout are anadromous, have a life cycle much like that of the salmon and suffer an appreciable post-spawning mortality, though the majority probably live long enough to reach the sea again. However, only a very small per cent return to the river for a second spawning (2). In contrast to the salmon and steelhead, the non-migratory rainbow trout appear to usually survive thieir initial reproductive effort. If activity of the adrenal plays an important role in the outcome of spawning we would expect to find histological differences in the cortical tissue of these three fishes. Indeed, such differences were found as will be presented in the following report. The biology of the salmon on its spawning migration has been discussed in earlier studies (3, 4) and described more fully by Greene (5).

MATERIALS AND METHODS

Source of material.—Three species of Pacific salmon were studied: (1) king (chinook) salmon (*Oncorhynchus tschawytscha*) were secured in Monterey Bay, in the open ocean near Bolinas Bay, and at various points from the mouth to the upper reaches of the Sacramento River, California.[3,4] The gonads of those captured in the sea were in the infantile state. In the river at points near the mouth and 285 miles upstream the gonads were found to be in varying stages of maturation, while at the spawning grounds 325 miles from the sea they were all sexually mature or nearly so. Spawning king salmon were also obtained at the Nimbus Hatchery on the American River, a tributary of the Sacramento and from the Entiat River, a tributary of the Columbia River 490 miles from the sea.[5] (2) Blueback (sockeye) salmon (*O. nerka nerka*) were taken at the Rock Island dam on the Columbia River not far below the Entiat River. These fish were within approximately 2 months of the expected spawning time. (3) Ripe and spent kokanee (*O. nerka kennerlyi*) were caught in Donner Lake, California. (This fish, an offshoot of the blueback salmon, has become land-locked, lives in lakes and spawns either in tributary streams or in the lake itself. The kokanee's life cycle is the same as that of its ancestral form, namely 4 years.)

Sea-run rainbow trout (steelhead) were secured from two stations on the Eel River, California, circa 82 and 154 miles from the sea and from several short coastal streams in the Santa Cruz Mountains of the same state. Rainbow trout, non-migratory, were obtained from the hatchery of Mr. George Dufour, Santa Cruz.[6] The adrenal tissues of

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[5] The authors are much indebted to Mr. Roger E. Burrows, Salmon Cultural Laboratory, U. S. Fish and Wildlife Service, at Entiat, Washington, for providing a considerable number of king and blueback salmon adrenals.

[6] We wish to thank Mr. George Dufour of Santa Cruz, California, for supplying us with many rainbow trout in various stages of sexual maturity.

63 king salmon, 21 blueback salmon, 28 kokanee, 25 steelhead and 36 rainbow (hatchery) trout were studied.[7]

Preparation of tissues.—The fish, lightly anesthetized in 1:20000 tricaine methanesulphonate, were killed by cutting the spinal cord just caudal to the head or were allowed to die in the anesthetic, or died of asphyxia. As far as could be determined the mode of death had no influence on the histological appearance of the adrenal tissue. The head kidney, which includes the arms and the upper pole of the cross (shown in Fig. 1), was separated from the kidney proper by a transverse incision at a point where the two arms of the cross join the body of the kidney, and dissected out immediately after death with blunt-nosed scissors. The tissue was placed in Bouin's solution where it was allowed to remain until histological preparation was begun. Hematoxylin and eosin was found to be a suitable stain. Other stains, Heidenhein's iron hematoxylin, Masson's trichrome, Gomori's aldehyde fuchsin and the PAS stain, added no additional information.[8] Since adrenal cortical cells of teleost fishes do not take fat stains or react to osmic acid fixation satisfactorily it was not possible to study the lipid content of the salmon adrenal tissue. In this connection we wish to thank Dr. Malcolm R. Miller of the Department of Anatomy, University of California Medical School, for preparing some osmic acid-fixed sections of salmon head kidney for us.

RESULTS

Location of the adrenal cortical tissue

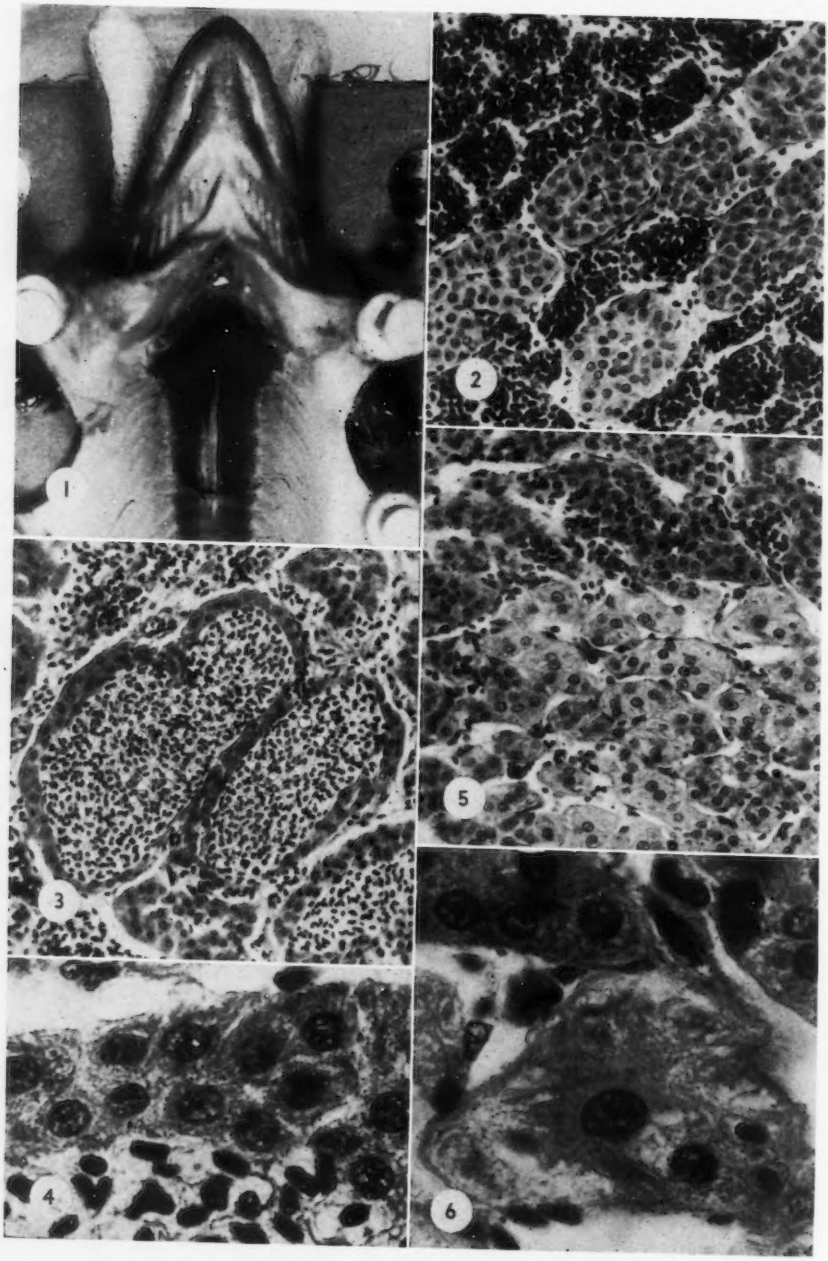
As in the case of most other teleost fishes, the adrenal cortical tissue of the salmonids is situated in the so-called head kidney (6). In the salmon and trout this structure lies dorsal to the heart and appears as a cross-shaped body (Fig. 1). The bulk of the adrenal tissue is found in a fairly narrow zone just cephalad of a line joining the tip of the cross arms. Giacomini (7) who gave the first account of the adrenal cortical tissue in salmonid fishes, including Atlantic salmon and trout, and later Fontaine and Olivereau (8) describe the cortical cells as occurring in small clumps distributed irregularly in the "lymphatic" tissue of the head kidney, either surrounding small branches of the cardinal vein or as discrete islets. This is in general the picture we find in Pacific salmon and rainbow trout. What Giacomini and others designated as lymphatic tissue in the head kidney we prefer to call hemopoietic, because of the active blood formation observable in this region of the kidney. However, since it seems probable that the blood cells are all derived from a primitive lymphocyte (9) this distinction may not be important.

Characteristics of the adrenal cortical tissue in salmon

1. *Sexually Immature Before Their Fluvial Migration.*—In king salmon

[7] For their valuable assistance in securing specimens on the field trips we are greatly obliged to Dr. Sydney F. Thomas, Dr. Marcus A. Krupp, Dr. Cutting B. Favour, Palo Alto Medical Research Foundation, Palo Alto, California; and Mr. Satoshi Hane, Metabolic Unit, University of California Medical Center, San Francisco, California.

[8] We are indebted to Mr. John Daniels of the Department of Medicine, University of Chicago, and to Miss Janice Doron of The May Institute, Cincinnati, for the excellent histological preparations on which this study is based.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(*O. tschawytscha*) with infantile gonads, captured in the sea, the adrenal cortical cells were found to occur in small clumps or cords usually adjacent to or surrounding a venule (Fig. 2). In certain fish these cells were present principally as narrow collars 1–3 cells thick around medium-sized veins (Fig. 3). The cells are characterized by a round nucleus, circa 5 μ in diameter containing fine reticular chromatin, a single nucleolus and a moderate amount of finely granular cytoplasm (Fig. 4).

2. *Sexually Maturing During Migration.*—Of three spring-run salmon taken in the lower reach of the Sacramento River (at Pittsburgh) in March, two showed hyperplasia of the adrenal cortical tissue as compared with salmon caught in the sea. These two fish exhibited beginning development of their gonads. In the fish without adrenal hyperplasia the testes were still in the infantile state. Salmon obtained in May at Mill Creek, a tributary of the Sacramento River some 285 miles upstream, all showed developing testes and ovaries though they were still far from mature. The testes weighed on the average one tenth of their mature weight and the ovaries one fifth. With a single exception the adrenal tissue of all the 26 fish examined at this station was found to be in a state of mild to moderate hyperplasia. This change consisted of increased thickness of the collars of cells surrounding the blood vessels, coalescence of the isolated cell clumps and cords characteristic of the marine fish with infantile gonads and increased size of many of the individual adrenal cells.

As sexual maturation proceeds, the degree of hyperplasia increases. Blueback salmon (*O. nerka nerka*) within two months of spawning and possessing well developed gonads, showed large masses of adrenal cells. The individual cells were for the most part about the size of those in the immature fish—nuclei 5 μ and total cell diameter 10–12 μ. However, here and there within the adrenal cell mass occurred clusters of much larger light-staining cortical cells with nuclei approximately 8 μ and total cell diameter up to 30 μ (Fig. 5). Their granules were dispersed and some areas of the

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Fig. 1. Rainbow trout. Heart and esophagus excised to show head kidney lying immediately dorsal to these structures. Kidney capsule removed part way. Slightly reduced.

Fig. 2. Adrenal cortical tissue of immature male king salmon captured in Monterey Bay. Cells arranged in cords lying in hemopoietic tissue of head kidney. ×225.

Fig. 3. Adrenal cortical tissue of immature male king salmon captured in brackish water near the mouth of the Sacramento River. Cells surround branches of the cardinal vein in form of narrow collars. ×225.

Fig. 4. Higher magnification of cells shown in fig. 3. Nuclei rich in chromatin and cytoplasm filled with fine granules. ×1000.

Fig. 5. Adrenal tissue of female blueback salmon with maturing ovaries. Area of large pale staining cells surrounded by smaller cells characteristic of the immature salmon adrenal. ×225.

Fig. 6. Higher magnification of cells shown at left margin of fig. 5. The very large cell is contiguous with two smaller ones. ×900.

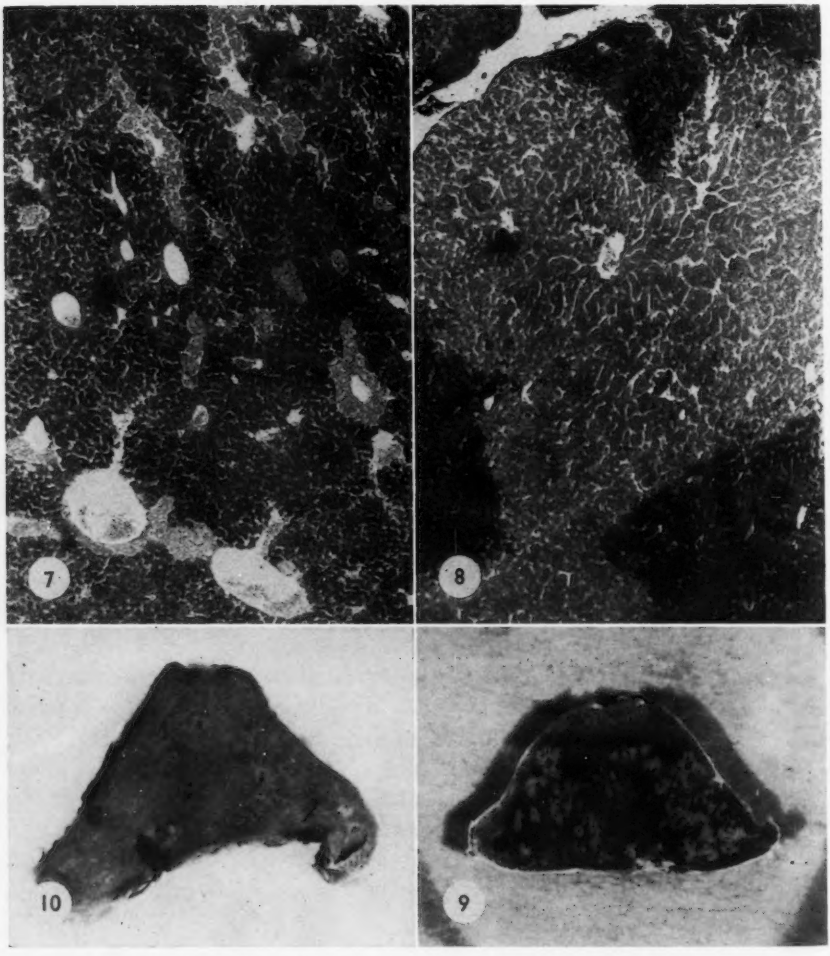

FIG. 7. Head kidney tissue of immature male king salmon taken in Monterey Bay, showing islets of adrenal cells mostly surrounding small blood vessels. ×36.

FIG. 8. Part of a single mass of hyperplastic adrenal cortical tissue present in spawning female blueback salmon taken at Entiat on the Columbia River. ×36.

FIG. 9. Slice of head kidney of spawning king salmon. Adrenal cortical tissue appears as whitish areas frequently surrounding blood vessels. ×1½.

FIG. 10. Slice of head kidney of immature king salmon. No adrenal cortical tissue visible grossly. ×1½.

cytoplasm were devoid of granules (Fig. 6). Mitoses were found occasionally in the cortical cells of fish with developing gonads.

3. *Sexually Mature and Spawning.*—By the time full sexual maturity had been reached, marked hyperplasia of the adrenal cortical tissue was found

in all the salmon studied. That the volume of this newly formed tissue must be many times that present in the immature fish is indicated by a comparison of Figures 7 and 8. The masses of cortical cells were often so large as to be visible grossly as irregular whitish areas in slices of Bouin's-fixed material (Fig. 9). A similar slice of head kidney from the same location in an immature salmon showed only the finely textured pattern of the hemopoietic tissue (Fig. 10). The cortical cells were arranged either in cords two to three cells thick or in larger lobulated groups. Separating these groupings were narrow spaces—sinusoids containing blood cells. The nuclei were quite uniform in size averaging 7 μ in diameter (Fig. 11). The total cell diameter varied from 10 μ to 18μ but most of the cells were in the larger category. The cytoplasm contained many evenly dispersed discrete granules as shown in Figure 12 and the cells were all of one type. Mitoses were rare in the sexually mature or spent fish.

In the great majority of the spawning salmon degenerative changes were observed in the cortical cells, sometimes generalized, more often focal, but in certain fish, even those which had spawned, these cells appeared to be in a healthy state. The nature of the deteriorative process appeared to differ in individual fish. Large hemorrhages were not infrequently seen in the hemipoietic tissue, extending sometimes into the cortical cell masses. Usually the process of degeneration was initialed by vascularization of the adrenal tissue. Newly formed blood channels broke up the cords and solid cell masses into small isolated groups or single cells (Fig. 13). Such cells on the periphery of the mass were seen to be disintegrating. Changes in the individual cells consisted in loss of the integrity of the granules which gave the cytoplasm a curdled appearance (Fig. 14) (a change not constantly present); vacuolization of the cytoplasm (Fig. 15); vacuolization of the nuclei; and as degeneration progressed, distortion of the nuclei, pycnosis, caryorrhexis, caryolysis and final dissolution of the cell (Fig. 16). There appeared to be little degeneration of the adrenal cells until sexual maturity had been reached.

The progressive changes in the adrenal cortical tissue from immaturity to the post-spawning state were essentially identical in all three species of salmon and no differences between males and females were detected.

The adrenal cortical tissue in rainbow trout

1. *Migratory (steelhead).*—Specimens of sexually immature adult steelhead trout were not available for study nor were we able to secure these trout in the earlier stages of sexual maturation.[9] However, the adrenal tissue of two juvenile hatchery-reared steelhead were examined and it was found to be indistinguishable from that of the non-migratory trout described below.

[9] Steelhead trout are rarely caught in the sea so practically nothing is known about their marine life.

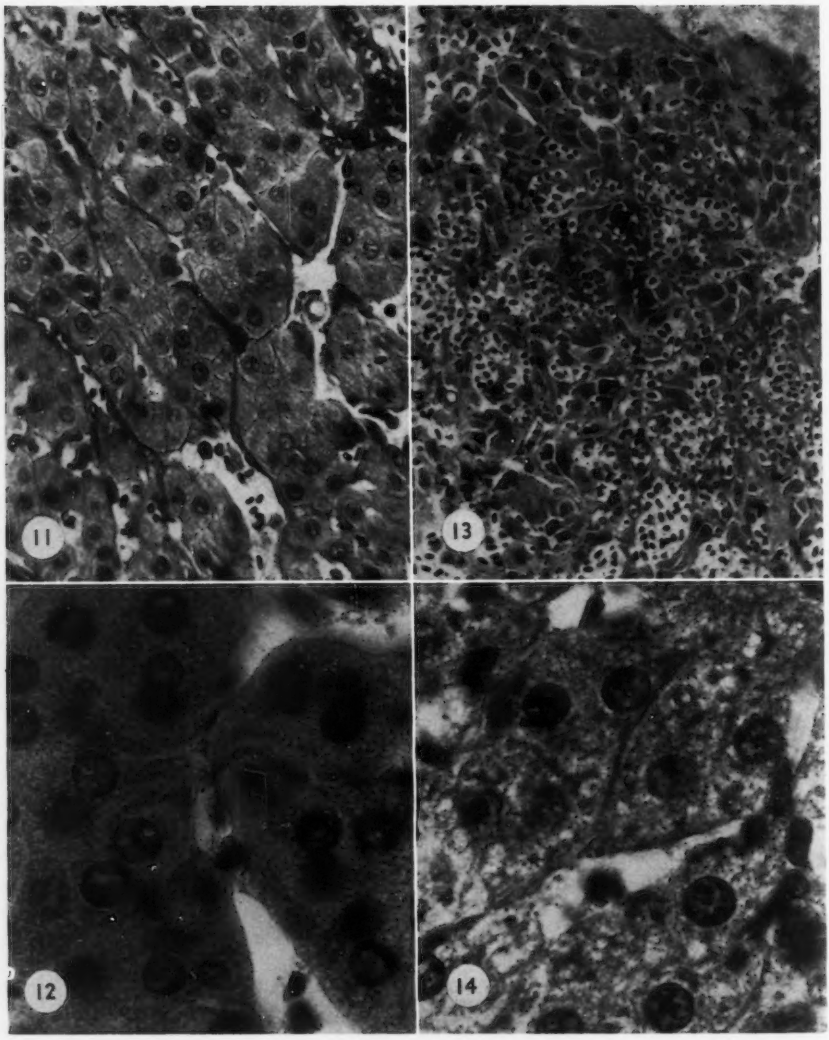

FIG. 11. Adrenal cortical tissue in a spawning female king salmon; cells, uniform in size and appearance, are arranged in cords separated by sinusoids. ×350.

FIG. 12. Adrenal cells in sexually mature female kokanee captured in Donner Lake. Cytoplasm filled with fine evenly distributed granules. ×1000.

FIG. 13. Adrenal cortical tissue of spawning female king salmon showing vascularization of tissue with degeneration of adrenal cells. ×200.

FIG. 14. Degenerative change in adrenal cells of spent female kokanee. Cytoplasm has curdled appearance. Contrast with fig. 12. ×1000.

Sexually mature and spawning steelhead trout exhibited extensive hyperplasia of the adrenal cortical tissue; in certain instances fully as marked as that occurring in spawning salmon (Fig. 17). The distribution of the cell masses, the arrangement of and character of the individual cells resembled in all respects the hyperplastic adrenal tissue in the salmon. However, the steelhead trout differed from the salmon in that quite a number of the adrenals examined (10 out of 25) showed no evidence of degeneration. This was true even of those fish held in captivity a week or ten days following spawning, which resulted in marked physical deterioration and a high mortality (2). The nature of the degenerative changes was similar to that occurring in the adrenal tissue of the spawning salmon, although the changes were usually less marked in degree. Also in contrast to the salmon, mitoses were found fairly frequently in the cortical cells of the steelhead; more often in the fish held after spawning than in those freshly caught.

2. *Non-migratory (hatchery trout).*—(*a*) *Sexually immature.*—Adrenal cortical cells were sparse. Histologically they resembed the cortical cells of the immature king salmon in the sea.

(b) *Sexually mature.*—Full sexual maturity and spawning was associated with some hyperplasia of the adrenal cortical tissue. This was very much less pronounced than occurs in spawning steelhead and salmon. (Figure 19 shows the distribution and extent of adrenal tissue in a ripe rainbow trout which should be compared with that of a spawning salmon shown on Fig. 8.) The adrenal tissue could not be detected macroscopically in slices of head kidney. The cortical cells showed relatively little change in size and character from the juvenile state, although in an occasional trout the cells were a little larger and exhibited more of the character of those occurring in steelhead trout and salmon at this stage. Degenerative changes were present in half the fish, but usually not severe. Occasionally the only evidence of deterioration was vacuolization of the nuclei (Fig. 18). In trout under 7 inches in length with precocious maturation of the testes, no hyperplasia of the adrenal tissue was evident.

Table 1 is a summation of the survival after spawning and associated adrenal changes in the Pacific salmon, steelhead trout, and non-migratory rainbow trout.

DISCUSSION

Very little is known concerning the role played by the adrenal gland in the body economy of teleost fishes. Presumably the fundamental function of the fish's adrenal cortical cells is to provide hormones for certain metabolic needs and to meet increased tissue requirements occasioned by stress as is known to occur in the higher vertebrates. That the adrenal cortical tissue does respond to certain stresses is indicated by Chavin's (10) experiments on the effect of placing xanthic goldfish in saline solutions. This re-

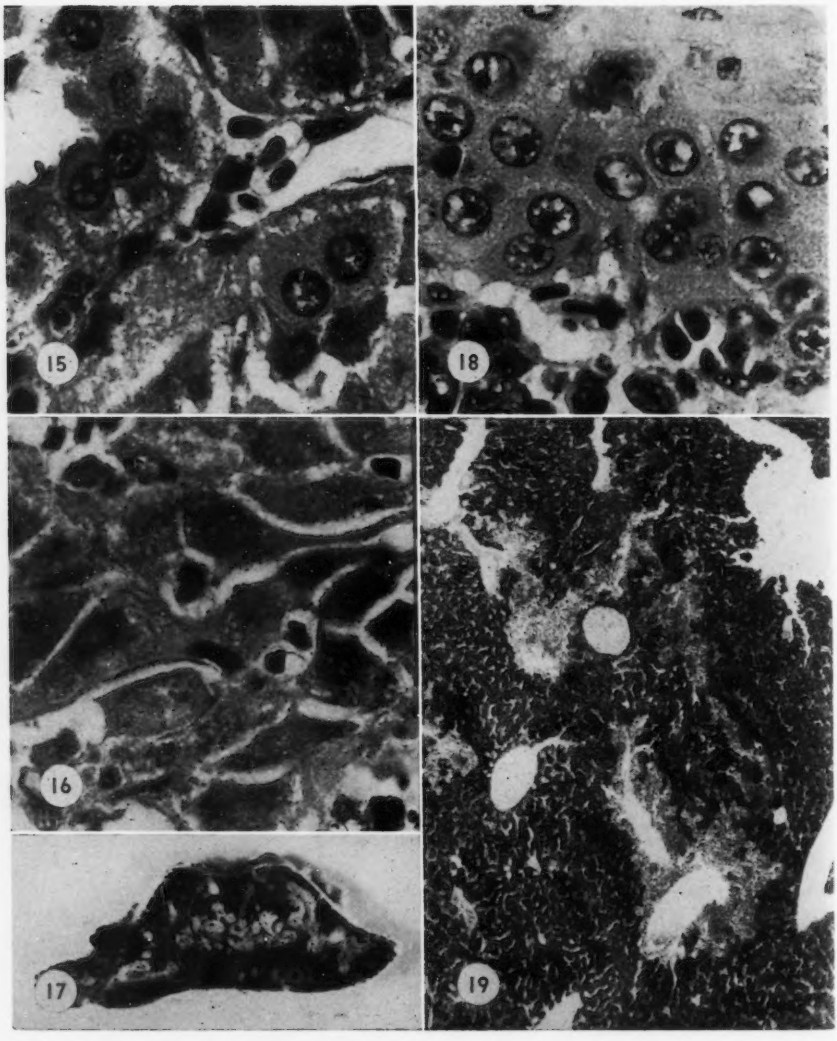

FIG. 15. Degenerative change in adrenal cells of spent male king salmon. Note especially vacuolization of cytoplasm. ×1000.

FIG. 16. Extensive degeneration of adrenal cells of spent female king salmon. Note pycnosis and distortion of nuclei and their final disappearance in many cells. ×1000.

FIG. 17. Slice of head kidney of a spawning female steelhead trout. Presence of abundant adrenal tissue indicated as white areas. ×1½.

FIG. 18. Adrenal cells in immature female rainbow trout showing marked vacuolization of nuclei with very little accompanying change in the cytoplasm. ×1000.

FIG. 19. Head kidney of spawning male rainbow trout 10 inches long and 2 years old. Some hyperplasia of adrenal tissue present. Compare with fig. 8. ×36.

sulted in the production of melanophores in the fishes' skin and an associated hypertrophy of the adrenal cortical cells. Injected ACTH produced the same effects. Fontaine and Olivereau (8) found what they considered histological evidence of increased secretory activity of the adrenal cortical cells in the salmon smolt—the developmental stage at which the young fish migrate from fresh water to salt water. Previously Fontaine and Hatey (11) had shown that the concentration of 17 hydroxycorticosteroids in the blood of salmon smo.t was much higher than in the preceding juvenile

TABLE 1. SURVIVAL AFTER SPAWNING AND ASSOCIATED ADRENAL CHANGES IN PACIFIC SALMON, STEELHEAD TROUT, AND NON-MIGRATORY RAINBOW TROUT

	Pacific salmon	Steelhead trout	Non-migratory rainbow trout
Survival after spawning	All die	Many die	Few die
Hyperplasia of adrenal cortical tissue	Marked	Marked	Relatively little
Degeneration of adrenal cortical tissue	Almost all fishes	Only part and less marked than in salmon	Only part and much less marked than in salmon

stage, the parr, which lives entirely in fresh water. They also found high concentrations of adrenal corticoids in maturing and spawning Atlantic salmon, but made no mention of hyperplasia of the adrenal tissue in these fish.

To what stimuli may we attribute the hyperplasia of the adrenal cortical tissue occurring in the migrating Pacific salmon and steelhead trout? The principal stresses which these fish undergo during their spawning migration are (1) adaptation from salt water to fresh water, (2) unusual physical exertion required to reach the spawning grounds and (3) the building of a very large mass of gonadal tissue under conditions of starvation. The first of these stresses can be eliminated as a dominant factor since the kokanee which spend all their lives in fresh water exhibit adrenal hyperplasia when sexually mature. Likewise the stress of migration is absent in the kokanee (of Donner Lake) as they spawn on the shores of the lake. Thus development of gonads while starving constitutes a stress factor common to the migrating salmon, the steelhead trout and the kokanee. This does not of course exclude the possibility of other causes of adrenal hyperplasia in these fish. However, since we have no knowledge of any such influence unassociated with the sexual cycle it has seemed to us worth-while to examine available information on the nature and effects of gonad maturation in the several salmonids of the present study.

In Table 2 data on weights of testes and ovaries at sexual maturity are shown. It should be noted that there is not much difference in gonad weights between king salmon and steelhead trout. The gonads of the latter

fish constitute an even greater percentage of the body weight than do those of the blueback salmon. The fact that the gonad masses of the blueback salmon (12) and rainbow trout are in general alike whereas adrenal hyperplasia is marked in the former and slight in the latter may signify the importance of the role of starvation in this process since the hatchery-reared rainbow trout continue to feed actively during sexual maturation.[11]

TABLE 2. RELATIVE WEIGHTS OF GONADS AT TIME OF SPAWNING

	Gonad weight per cent of body weight			
	Ovaries	Number of fish	Testes	Number of fish
King salmon—Green (5)	25.0	(?)	—	
King salmon—Robertson & Wexler	21.2	(15)	4.6	(22)
			5.5[10]	(12)
Steelhead trout—Robertson & Wexler	19.0	(19)	4.0	(13)
Rainbow trout—Robertson & Wexler non-migratory	14.0	(13)	5.0	(15)
Blueback salmon—Idler and Tsuyuki (12) (*O. nerka*)	13.7	(55)	3.3	(25)
Blueback salmon—R. E. Burrows[12]	20.8	(15)	—	

Greene (13) showed that king salmon taken on the spawning grounds at the head waters of the Sacramento River had lost 50% of their wet muscle and 74% of their dry muscle weight as compared with salmon of similar length caught in the sea. The muscle protein of the spawning salmon was reduced by 30% (14). In their studies of sockeye salmon (*O. nerka*) on their spawning migration in the Fraser River, Idler and Tsuyuke (15) found that the spawning males had lost 35 per cent and the females 17% of their muscle protein. Whether analogous losses of muscle weight and muscle protein occur in the sedentary fresh water kokanee or in salmon which migrate only a short distance up coastal streams is not known nor have we any information on this subject in spawning steelhead and rainbow trout.

If the excessive depletion of body tissues caused by starvation and massive gonad building does act to incite hyperplasia of the adrenal cortical tissue in the salmon, it seems unlikely that this is the only stimulus since

[10] Testes of salmon caught several weeks before spawning. Mature males usually begin to shed milt before they reach the spawning ground.

[11] It is possible that the hatchery-reared rainbow trout employed in this study responded to the stress of spawning in a manner different from that of the wild variety, so that for an entirely satisfactory comparison of adrenal gland activity in spawning salmon and non-migratory trout, observations on a wild population of the latter would be necessary.

[12] We wish to thank Mr. Roger E. Burrows, who at our request made these weighings on blueback salmon reaching full maturity in the holding ponds of the Salmon Cultural Laboratory at Entiat.

adrenal hyperplasia was observed to begin while the gonads were in the early stages of development when the fat (and probably protein) stores were still abundant.

Some information bearing on this subject came from observations made by one of the authors (16) on gonadectomy in kokanee. A number of male kokanee castrated at 2 years of age showed regeneration of gonadal tissue after 1–2 years. These fish developed the signs of the sexually mature salmon, hooked jaw, humped back, eroded skin, and all died. The regenerated testicular tissue was found to be very limited in extent, often only a single piece weighing a few hundred milligrams, whereas the fully mature testis of a normal kokanee weighed 10–15 gm. The adrenal tissue of these fish showed well marked hyperplasia. Castrated kokanee without gonad regeneration lived much longer and showed no adrenal hyperplasia.

The distinctive character of the three stages in the evolution of the adrenal cortical cells at successive stages of sexual maturation suggests certain analogies to the several cell types in the mammal. The relatively small, rather densely granulated cell present in the sexually immature salmon bears some resemblance to the cells of the mammalian zona glomerulosa. The larger cell and cord arrangement of the hyperplastic adrenal certainly suggests the fasciculata type while the last or degenerative stage might be taken to represent the reticularis zone.[13]

A relationship between adrenal hyperplasia and survival after spawning is only partially elucidated by this study. Contrast between the marked hyperplasia in the salmon which all die and the small degree of adrenal hypertrophy in the non-migratory rainbow trout which survive, seems clear enough. But the lack of any conspicuous difference in adrenal reactivity between salmon and steelhead trout (most of which probably recover from the immediate effects of their initial reproductive effort) makes it difficult to interpret the disparity in post-spawning survival of these two fishes on this basis.

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[13] The morphological arrangement of the mammalian adrenal cortex into a zona glomerulosa, zona fasciculata, and zona reticularis has also been correlated with a functional capacity for each zone. For example, the zona glomerulosa produces mineralocorticoids, the zona fasciculata secretes glucocorticoids, and the zona reticularis or zone of "senescence" the sex steroids. For an excellent review of the morphologic and functional interrelationships of the adrenal cortex the reader should consult: Greep, R. O. and H. W. Deane: Ann. N. Y. Acad. Sci. 50:596. 1949.

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GLOMERULOTROPIC ACTIVITY OF AN ACETONE EXTRACT OF PINEAL TISSUE[1]

GORDON FARRELL[2]

Department of Physiology, Western Reserve University, Cleveland, Ohio

ABSTRACT

A crude acetone-soluble fraction of beef pineal tissue was assayed for steroidogenic activity in the decerebrate dog. Aldosterone secretion was markedly stimulated without a significant effect on cortisol output. When the material was partitioned between water and hexane, the hexane fraction was found to contain the activity. The finding is taken as additional evidence for the existence of glomerulotropin, a specific hormone for aldosterone secretion, in pineal tissue or an adjacent structure.

NEUTRAL saline extracts of pineal tissue have been found to selectively stimulate aldosterone secretion, when assayed in the decerebrate dog (1, 2). This observation offered support for the concept of a specific factor for aldosterone secretion ("glomerulotropin") presumably secreted either by the pineal body or by an adacent structure included in the extracts. In the present study a crude commercially prepared acetone extract of pineal tissue was assayed for steroidogenic activity in the decerebrate dog. Aldosterone secretion was markedly stimulated in the absence of a significant effect on cortisol secretion. These experiments are taken as additional evidence for the existence of glomerulotropin, and again implicate the epiphysis cerebri as the source of the hormone.

METHODS

The extracts of beef pineal tissue were prepared by the Wilson Laboratories of Chicago, Illinois.[3] Freshly obtained pineal glands were accumulated in a large excess of absolute acetone. The acetone insoluble material from the glands was subsequently removed and ground to yield a light pink powder. The supernatant was concentrated in vacuum at 30° or less to remove the acetone. The residue consisted of a reddish-brown aqueous slurry, which will be referred to as the "acetone-soluble fraction."

For assay, samples of each material were dissolved in 500 ml. 0.9% sodium chloride and infused intravenously. The assay for glomerulotropic activity employs the midcollicular decerebrate dog, in which the brain substance, including the pituitary and

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[2] Work carried out during the tenure of an Established Investigatorship of the American Heart Association.

[3] We are grateful to Dr. C. E. Graham for his assistance in the preparation of this material.

pineal glands, are removed with suction (3). Three to four hours after decerebration the left lumboadrenal vein is cannulated for collection of adrenal venous blood and the solution to be assayed is infused during the collection period (1½ to 2 hours). Aldosterone and cortisol are isolated from the adrenal venous blood by methods described in detail in earlier reports (3, 4). Each experiment yields an estimate of the cortisol secretion rate as well as the aldosterone secretion rate. One dog serves to assay a single sample; replicate experiments are carried out to obtain estimates of error.

RESULTS AND DISCUSSION

1. Acetone-soluble fraction

Two lots of this material were assayed, each in duplicate. The first lot consisted of about 35 ml. of slurry, obtained from 57 grams of fresh pineal tissue. Two samples of 7 ml. each were taken for assay. The second lot consisted of approximately 2500 ml. of similar material. Two samples of approximately 5 ml. each were assayed. Within the limits of error of the assay method, the activity of the two lots appeared to be the same, and the results were combined for statistical evaluation (Table 1). The cortisol secretion rate was unchanged from the level in decerebrate controls. On the other hand, the extract induced highly significant stimulation of aldosterone secretion ($P < .001$), to values approximately those found in intact animals (Table 1).

TABLE 1. STEROIDOGENIC ACTIVITY OF PINEAL EXTRACTS

Experiment	No. of Expts.	Cortisol μg./kg. body wt./hr.	Aldosterone μg./100 Kg. body wt./hr.
Intact controls	9	32.8 ±3.9*	31.2 ±3.7
Decerebrate controls (saline-infused)	8	2.19±0.49	7.86±0.87
Decerebrate, infused with pineal extracts			
Acetone-soluble fraction	4	3.98±1.03	25.0 ±4.15**
Hexane-water partitioned acetone-soluble fraction			
Hexane fraction	4	3.69±0.66	34.6 ±4.2**
Aqueous fraction	3	9.93±3.0	10.92±3.2
Acetone-insoluble fraction	2	34.2, 26.3	18.8, 31.0

* Mean and standard error.
** Statistically significant ($P < .001$) when compared with decerebrate controls.

Aliquots (5 to 15 ml.) of the second lot of the acetone-soluble fraction were diluted with 50 ml. water and washed 6 times with 50 to 100 ml. of hexane. In 3 such experiments, the aqueous fraction was assayed and found to be virtually free of glomerulotropic activity. A trace of ACTH activity was present as indicated by the increased secretion of cortisol (Table 1). In four experiments, the hexane fraction was evaporated to dryness under a stream of filtered air, dissolved in a small volume of ethanol and taken up in saline for assay. This material stimulated aldosterone secretion (Table 1). The results strongly suggest that the active factor is lipid soluble. The hexane fraction did not stimulate cortisol output to any significant degree, again indicating virtually complete absence of ACTH (Table 1).

The results presented here are of a qualitative rather than a quantitative nature, and no statement as to the amount of the hormone in the extracts is possible. However, the presence of a factor which stimulates aldosterone secretion and which is quite distinct from ACTH is clearly indicated. Earlier observations indicated the probable existence of such a factor (5, 6, 7).

2. Acetone-insoluble fraction

A sample of the powder prepared from acetone-washed pineal glands weighing 2.6 grams (equivalent to approximately 16 grams of fresh pineal tissue) was divided into equal lots and assayed in duplicate (Table 1). Aldosterone secretion was increased; however, there was sufficient ACTH present in the sample to induce a significant increase in cortisol secretion. The presence of ACTH activity in this material is unexplained, although contamination in the process of collection is a possibility.

In the present study, it appears that the corticotropic activity present in commercially collected pineal tissue remains with the defatted residue. On the other hand, glomerulotropin appears to be soluble in the acetone supernatant, and to preferentially go into hexane in hexane-water partitioning. Speculation as to the chemical nature of the hormone is scarcely justified at this stage of development of the problem, except for the obvious possibility that it is a lipid, or is associated with a lipid soluble substance.

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METABOLISM OF MONOIODOTYROSINE, DIIODOTYROSINE, TRIIODOTHYRONINE AND THYROXINE BY L-AMINO ACID OXIDASE[1]

MINORU NAKANO, T. S. DANOWSKI AND ATSUSHI UTSUMI

Section of Endocrinology and Metabolism, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

ABSTRACT

Thyroxine, triiodothyronine, diiodotyrosine and monoiodotyrosine, labelled with I^{131}, were incubated with L-amino acid oxidase of cobra venom, and their metabolites studied by paper chromatography and radioautography. Under these conditions, the iodinated tyrosines were converted to the corresponding alpha-keto acid and acetic acid, inorganic iodide, and a number of unknown compounds. The deiodination of iodotyrosines by L-amino acid oxidase occurred after their corresponding alpha keto acids were formed by deamination of the amino acids. Triiodothyronine and thyroxine were converted to their respective acetic acid derivatives, but not to inorganic iodide. The rate of degradation of these aromatic iodinated compounds by L-amino acid oxidase was related to the number of iodine atoms in the phenyl or diphenyl ether radical; i.e. monoiodotyrosine→diiodotyrosine→triiodothyronine→thyroxine.

TONG *et al.* (1) demonstrated that I^{131}-labelled diiodotyrosine is converted to 3,5-diiodo-4-hydroxyphenylpyruvic acid and inorganic iodide by snake venom and have suggested that this is similar to the metabolism of diiodotyrosine by rat kidney and liver. Roche *et al.* (2) found radioactive triiodothyropyruvic acid and tetraiodothyropyruvic acid in urine and bile of thyroidectomized rats after the injection of I^{131}-labelled triiodothyronine and thyroxine, respectively. They also have reported the presence of triiodothyroacetic acid in kidney of rats treated with I^{131}-labelled triiodothyronine (3). Recently, Tomita *et al.* (4, 5) have found that triiodothyronine and thyroxine labelled with I^{131} were converted to their corresponding acetic acid analogues by mitochondria prepared from rat kidney cells.

These results raise the possibility that the metabolic pathway of thyroid hormones and their related compounds is in part dependent upon L-amino acid oxidase present in animal tissue. The present work was undertaken to investigate the deamination of thyroid hormones and related compounds by using L-amino acid oxidase of cobra venom.

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MATERIALS AND METHODS

A. *In vitro Experiments.*

Cobra venom[2] was used as a source of L-amino acid oxidase without purification. Twenty milligrams of the cobra venom were dissolved in 20 cc of a 0.05 M. phosphate buffer containing 0.077 M. of KCl (pH 7.25).[3] One ml. of this solution was added into tubes containing known amounts of the radioactive iodine compound. The reaction mixture was immediately incubated in a constant temperature water bath (38° ± 0.5°) for various time intervals. At the end of incubation, the mixtures were quickly cooled with ice and brought to a pH of 11.0 with one drop of 2N NaOH, to stop the enzyme reaction. The zero time sample was obtained by cooling and alkalizing immediately after mixing the iodinated amino acid with the cobra venom.

B. *Separation of the Products.*

Approximately 30 μl. of each reaction mixture were placed on Whatman No. 1 filter paper and chromatographed in two solvent systems: collidine-water (100:35.5) in ammonia atmosphere for the separation of the products of monoiodo- and diiodotyrosine, and tertiary amyl alcohol saturated with 2N NH_4OH for the metabolites of thyroxine and triiodothyronine. The filter paper was dried in air and radioautographs were obtained. The position of the carrier compounds, added as a marker to the reaction mixture prior to chromatography, was located by color development with various reagents: 2,4-dinitrophenylhydrazine in HCl for the corresponding alpha keto acids, while diazotized sulfanilic acid was used for all the other compounds. Areas which coincided with black bands of radioautograms were cut out and counted with a Geiger-Muller counter.

C. *Preparation of Radioactive Iodine Compounds.*

The compounds were prepared by iodination of L-tyrosine (Matheson Company) or L-3,5-diiodothyronine (Smith, Kline and French Laboratories) with a mixture of I^{131} and inorganic iodine I_2^{127} as described by Roche *et al.* (3, 6). Both monoiodo- and diiodotyrosine were obtained when 75 μl. of L-tyrosine solution (18 mg. of tyrosine in 20 cc of concentrated ammonia) were iodinated with a mixture of 40 μl. of iodine solution (189 mg. of I_2 in 60 ml. of ethanol) and 60 μl. of Na I^{131} (6.3 mc.)

Also, both triiodothyronine and thyroxine were formed when 45 μl. of 3,5-diiodothyronine solution (25.6 mg. of L-diiodothyronine in 10 cc of concentrated ammonia) were iodinated with a mixture of 30 μl. of the iodine solution described above and 60 μl. of Na I^{131}(14.8 mc.). The first two of these four compounds were purified by paper chromatography in acetic acid-water-n-butyl alcohol (15:35:100); similarly, the last two were chromatographed in dioxan-n-butyl alcohol-2N NH_4OH (1:4:2) and then in tertiary amyl alcohol saturated with 2N NH_4OH. The following specific activities were obtained just before the incubation experiments: 17.5 μc/gamma for monoiodotyrosine, 15 μc /gamma for diiodotyrosine, 13.5 μc/gamma for triiodotyronine, and 8.3 μc/gamma for thyroxine.

D. *Preparation of the Non-Radioactive Iodine Compounds.*

The following methods were used: monoiodotyrosine by Pitt-Rivers' procedure (7),

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[2] This enzyme was obtained from Hynson, Westcott and Dunning, Inc., Baltimore. It is stated to contain a catalase in addition to L-amino acid oxidase.

[3] In accord with the observations of Kearney and Singer (Arch. Biochem. 33: 377, 1951), KCl was added in order to protect the enzyme from spontaneous inactivation.

both acetic and pyruvic acid analogues of monoiodotyrosine by our method,[4] and both acetic and pyruvic analogues of diiodotyrosine as described by Papa *et al.* (8) and Tong *et al.* (1) respectively. The remainder were obtained from commercial sources.

RESULTS

A. *Metabolism of I^{131}-Labelled Monoiodotyrosine.*

In Figure 1 the products formed from I^{131}-labelled monoiodotyrosine by incubation with crude l-amino acid oxidase are shown. In addition to the monoiodotyrosine and inorganic iodide bands, four other bands appeared in all radioautographs of the paper chromatograms. Of these, two coincided with the color band formed by added 3-monoiodo-4 hydroxyphenylpyruvic acid and by acetic acid, respectively. When the elutants of these and the other two bands were re-chromatographed in dioxane-n-butyl alcohol-2N ammonia (1:4:2), these were found to have Rf values of 0.67 and 0.36 respectively, coinciding with those of added pyruvic acid and acetic acid derivatives of monoiodotyrosine. The other bands were named MX_1 and MX_2, respectively, without identification.

Figure 2 shows the rate at which the metabolites are formed from monoiodotyrosine by cobra venom. This iodinated amino acid was rapidly converted into 3-iodo-4 hydroxyphenylpyruvic acid with 60 per cent of the total radioactivity within 15 mintues. However, 3-iodo-4-hydroxyphenylpyruvic acid then decomposed rapidly after reaching a peak, with an increase in inorganic iodide. The other iodinated compounds, such as 3-iodo-4-hydroxyphenyl acetic acid, the compound located at the chromatographic origin, and MX_{1-2} all increased linearly with time up to the 20 minute point, and then reached a constant plateau.

B. *Metabolism of Diiodotyrosine.*

When this iodinated amino acid was incubated with cobra venom, it was converted to 3,5-diiodo-4-hydroxyphenylpyruvic acid, 3-iodo-4-hydroxyphenylpyruvic acid, as well as an unknown compound. The chromatogram of these metabolites is shown in Figure 3. The elutants of the radioactive bands corresponding to acetic and pyruvic acid derivatives of diiodotyrosine re-chromatographed in dioxane-n-butyl alcohol-2N NH_4OH (1:4:2) were found to have an Rf of 0.20 and 0.81 respectively, coinciding with those of the known compounds. 3-iodo-4-hydroxyphenylacetic acid was not found in the radioautographs of any samples.

As shown in Figure 4, the diiodotyrosine decreased to less than 20 per

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[4] Unpublished work. Analyses of the acetic and pyruvic acid analogues of monoiodotyrosine synthesized in this laboratory showed:

Acetic acid analogue: m.p. 183° (decomp.) (Found: C, 32.6; H, 3.4; I, 43.4. $C_8H_7O_3I \cdot H_2O$ requires C, 32.5; H, 3.1; I, 42.9%)

Pyruvic acid analogue: m.p. 168° (decomp.) (Found: C, 35.1; H, 2.6; I, 40.6. $C_9H_7O_4I$ requires C, 35.3; H, 2.3; I, 41.5%)

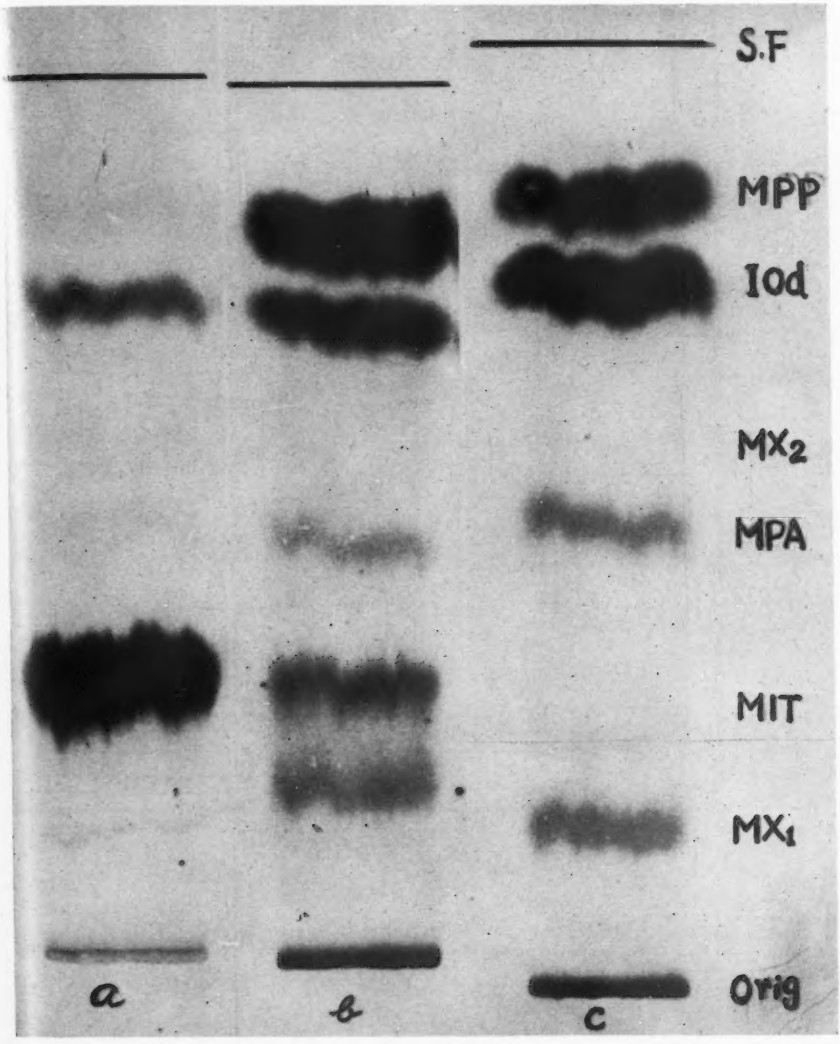

FIG. 1. Radioautograph of Chromatogram in Collidine-Water (100:35.5) in NH_3 Atmosphere, Showing Metabolites of I^{131}-Labelled L-Monoiodotyrosine (9.0×10^{-6} M) Produced by L-Amino Acid Oxidase.

Incubation interval of a: zero; b: 10 minutes; and c: 80 minutes.

MIT = monoiodotyrosine, Iod. = inorganic iodide. MPP = 3-iodo-4-hydroxyphenylpyruvic acid. MPA = 3-iodo-4-hydroxyphenylacetic acid. MX_1 and MX_2 = unknown compounds. SF = solvent front. Orig. = origin of paper chromatographs.

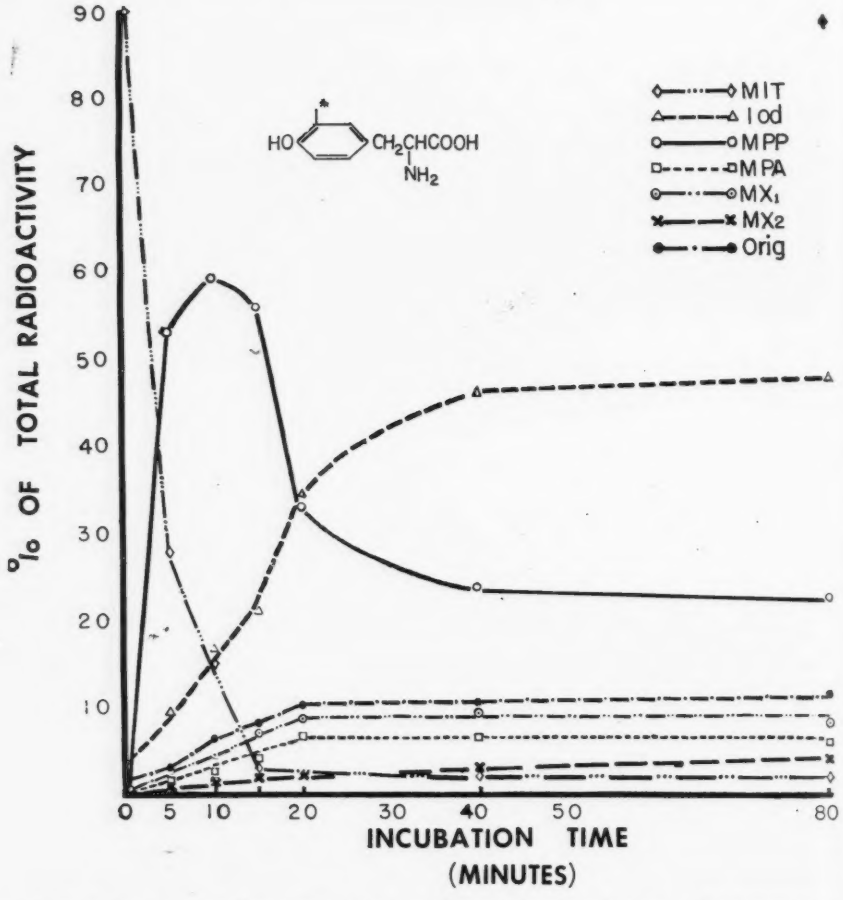

FIG. 2. Radioactive Fraction at Various Times After Incubation of Radioactive L-Monoiodotyrosine (9.0×10^{-6} M) with L-Amino Acid Oxidase.

Symbols as in Figure 1. ◆ Percent of monoiodotyrosine at 80 minutes after incubation of its acid with denatured enzyme obtained by boiling for 10 minutes.

* Position of radioactive iodine.

cent of the original amount within 40 minutes. On the other hand, 3,5-diiodo-4-hydroxyphenylpyruvic acid formed from diiodotyrosine reached a peak 30 minutes after incubation, and then decreased as inorganic iodide increased. The other iodine compounds increased linearly with time up to 40 minutes and then plateaued as did the metabolites of monoiodotyrosine. However, the conversion rate of diiodotyrosine to its metabolites, namely, to the corresponding alpha keto acid and inorganic iodide, was slower than that of monoiodotyrosine.

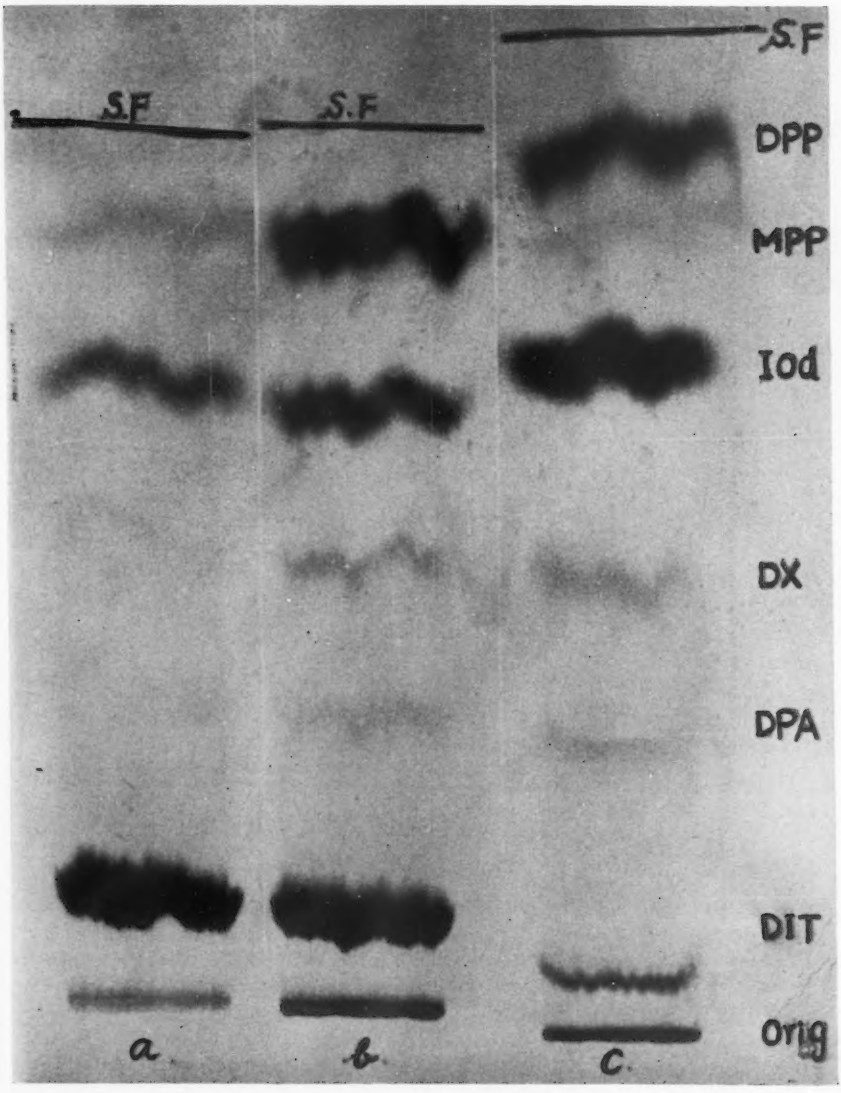

FIG. 3. Radioautograph of Chromatogram in Collidine-Water-NH_3 System, Showing the Metabolites of Radioactive L-Diiodotyrosine (8.2×10^{-6} M) Produced in the Presence of L-Amino Acid Oxidase.

Incubation interval of a: zero, b; 15 minutes; and c: 80 minutes.

DIT = diiodotyrosine. DPP = 3,5-diiodo-4-hydroxyphenylpyruvic acid. DPA = 3,5-diiodo-4-hydroxyphenylacetic acid. DX = unknown compound. See Figure 1 for other abbreviations.

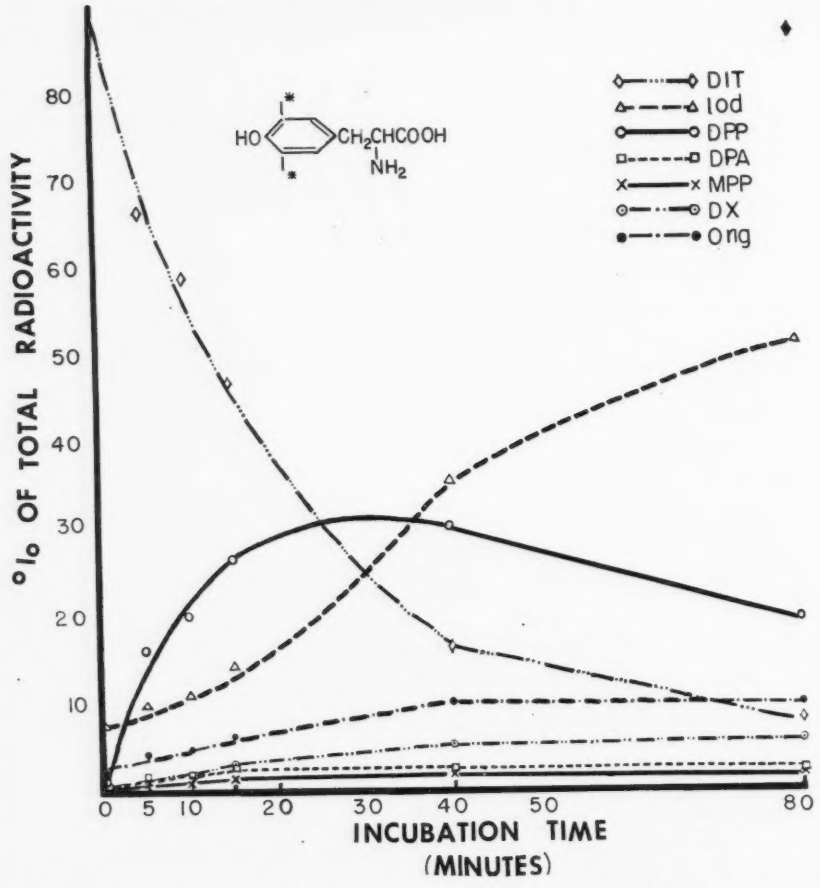

FIG. 4. Degradation of Radioactive L-Diiodotyrosine (8.2×10^{-6} M) by L-Amino Acid Oxidase.

Symbols as in Figure 3. ◆ Percent of diiodotyrosine at 80 minutes after the incubation of its acid with denaturated enzyme obtained by boiling for 10 minutes.

* Position of radioactive iodine.

C. *Metabolism of 3',3,5-triiodothyronine.*

I^{131}-labelled triiodothyronine used in this experiment still contained a relatively high amount of inorganic iodide, small amounts of an unknown compound, and triiodothyroacetic acid, despite attempts at purification by re-chromatography.

Figure 5 shows the chromatogram of the metabolites of triiodothyronine obtained with cobra venom. In addition to the intense black band of triiodothyronine and inorganic iodide, there were two bands which gradually

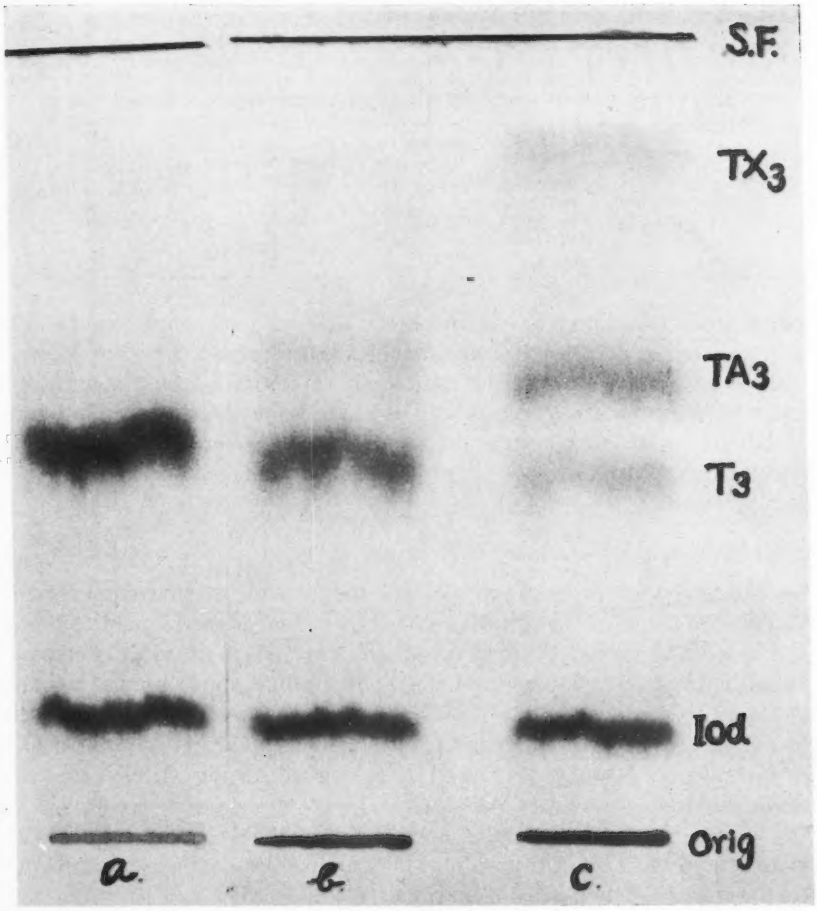

FIG. 5. Radioautograph of Chromatogram in Tertiary Amyl Alcohol Saturated with 2N NH_4OH, Showing the Metabolites of Radioactive L-Triiodothyronine (2.4×10^{-6} M), Obtained with L-Amino Acid Oxidase.

Incubation interval of a: zero; b: 40 minutes, and c: 80 minutes.

T_3 = 3′,3,5-triiodothyronine. TA_3 = 3′,3,5-triiodothyroacetic acid. TX_3 = unknown compound. Iod. etc.—see Figure 1.

increased in intensity with time. One of the bands coincided exactly with the colored band of triiodothyroacetic acid employed as carrier in paper chromatographs. This band was further identified by co-crystallization with synthesized triiodothyroacetic acid, as shown in Table 1. It seems likely, therefore, that most of the iodinated compound contained in the band was triiodothyroacetic acid. The unknown compound, named TX_3, was not identified. Figure 6 shows the rate at which these metabolites were

TABLE 1

21.2 mg. of non-radioactive 3′,3,5-triiodothyroacetic acid and the elutant of the radioactive compound from the paper were mixed, and crystallized four times from ethanol by dilution with water. The amount and the radioactivity of crystals were determined by means of microbalance and scintillation counter, respectively, at each stage of crystallization.

Stage of crystallization	Counts/minute/μg.
Original mixture	3.90
1st cryst.	3.42
2nd cryst.	3.50
3rd cryst.	3.47
4th cryst.	3.49

formed from triiodothyronine under the influence of cobra venom. The triiodothyronine decreased to one-half its initial amount within 40 minutes and reached a level lower than that of the triiodothyroacetic acid within 80 minutes after incubation. Both unknowns and the compound located at the origin of chromatograph showed a similar increase. The degradation of triiodothyronine proceeded at a rate slower than that of diiodotyrosine.

D. *Metabolism of I^{131}-Labelled Thyroxine.*

In Figure 7 the chromatogram of the metabolites of thyroxine formed under the influence of snake venom are shown. One of the bands was found in all radioautographs obtained from each reaction mixture at successive incubation times. The intensity of this band gradually increased. The band was found to coincide with color formed by carrier tetraiodothyroacetic acid on the paper chromatograph. It was further identified by paper elution with alcohol in water (1:1) and by co-crystallization with synthesized tetraiodothyroacetic acid (Table 2).

On the basis of these results, this band is believed to be tetraiodothyroacetic acid. Triiodothyronine and triiodothyroacetic acid were not detected in any of the radioautographs.

The degradation rate of the thyroxine, as shown in Figure 8, proceeded very slowly and amounted to no more than 15 per cent within 80 minutes after incubation. Consequently, the formation of tetraiodoacetic acid was only seven per cent at this time. There was no evidence of the formation of inorganic iodide from thyroxine, as in the case of triiodothyronine.

TABLE 2

18.3 mg. of non-radioactive tetraiodothyroacetic acid and the elutant from the paper chromatogram were mixed, crystallized, weighed and counted as described in legend for Table 1.

Stage of crystallization	Counts/10 minutes/μg.
Original mixture	5.15
1st cryst.	4.65
2nd cryst.	4.48
3rd cryst.	4.51
4th cryst.	4.56

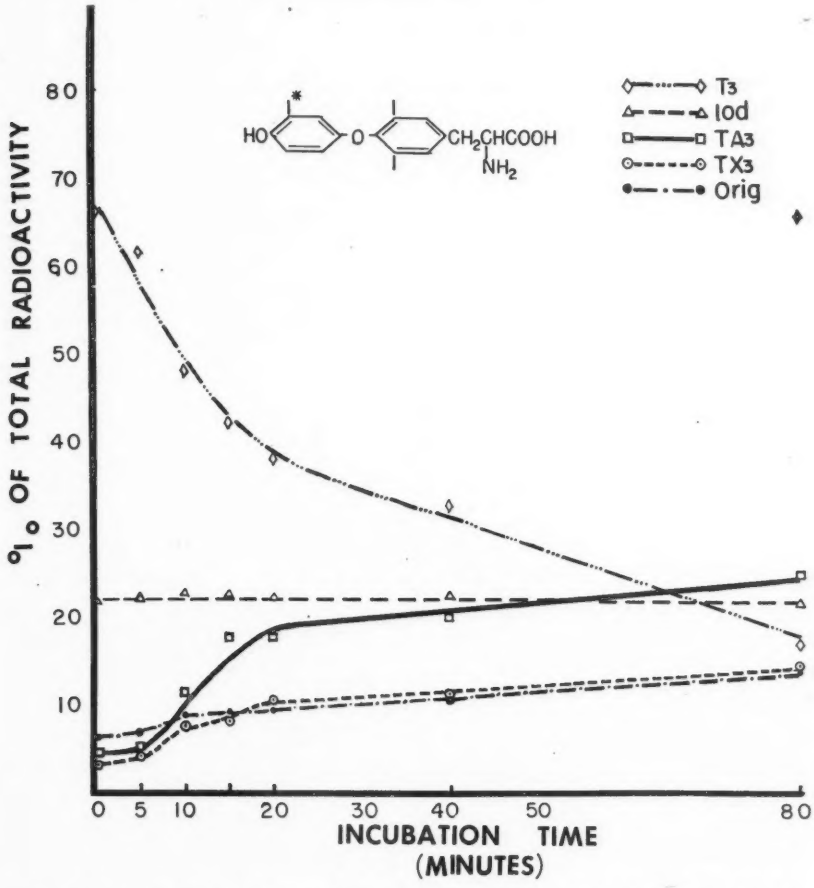

FIG. 6. Degradation of I^{131}-Labelled L-Triiodothyronine (2.4×10^{-6} M) After Incubation With L-Amino Acid Oxidase.

Symbols as in Figure 5. ◆ Percent of triiodothyronine at 80 minutes after incubation of its acid with denatured enzyme by boiling for 10 minutes.

* Position of radioactive iodine.

DISCUSSION

It has been shown that crude cobra venom contains several enzymes: catalase (9), phospholipase (10), adenosine-triphosphatase (11), and acetylcholine esterase (12) in addition to L-amino acid oxidase. Of these, catalase may decompose any hydrogen peroxide which might be formed from the iodinated amino acids by L-amino acid oxidase and thereby inhibit secondarily the formation of the corresponding acetic acids from the corresponding alpha keto acids by hydrogen peroxide. However, under the condition of our experiments it is probable that L-amino acid oxidase is the only enzyme acting upon the iodinated amino acids.

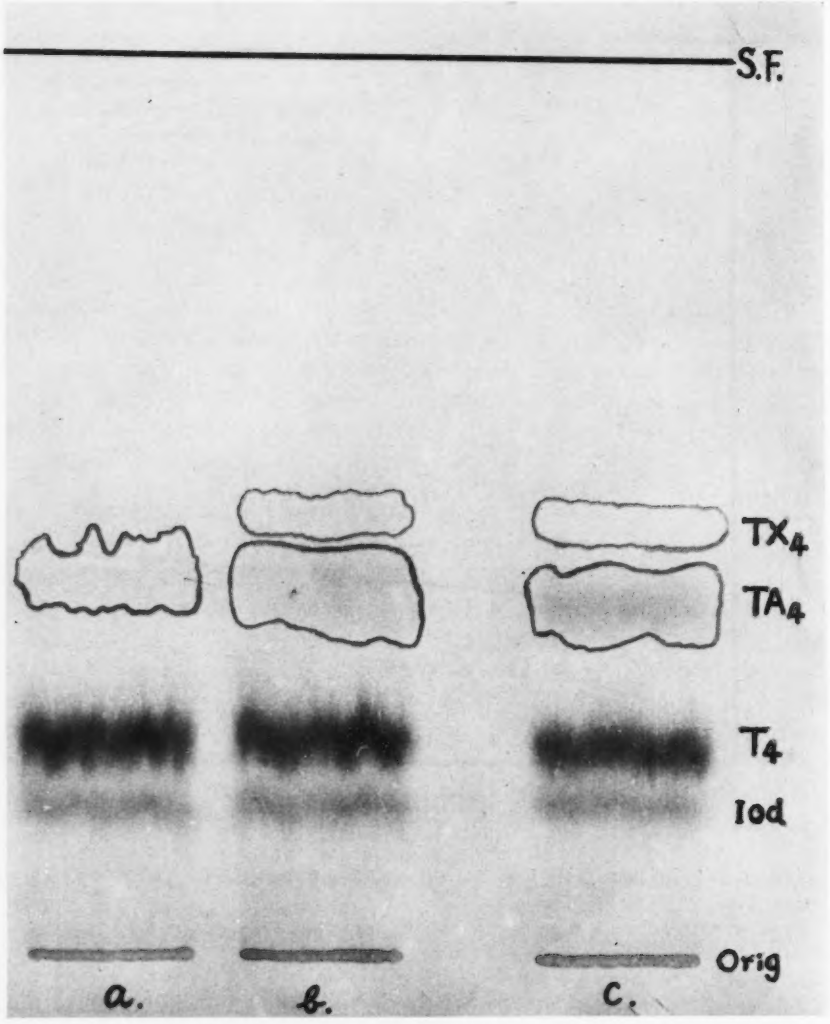

FIG. 7. Radioautographs of Chromatograms in Tertiary Amyl Alcohol Saturated with 2N NH_4OH, Showing the Metabolites of Radioactive L-Thyroxine (1.2×10^{-6} M) Obtained with L-Amino Acid Oxidase.

Incubation interval of a: zero; b: 40 minutes, and c: 80 minutes.

T_4 = thyroxine. TA_4 = tetraiodothyroacetic acid. TX_4 = unknown compound. See Figure 1 for other symbols.

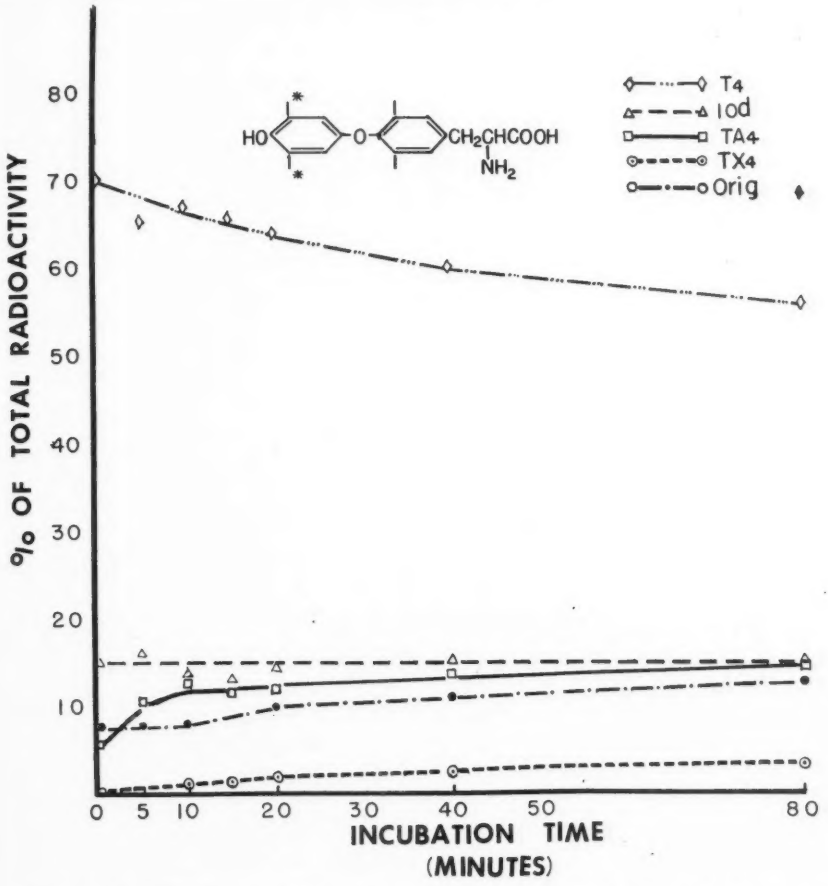

FIG. 8. Degradation of I^{131}-Labelled L-Thyroxine (1.2×10^{-6} M) by l-Amino Acid Oxidase.

Symbols as in Figure 7. ◆ Percent of thyroxine at 80 minutes after incubation of its acid with denaturated enzyme obtained by boiling for 10 minutes.

* Position of radioactive iodine.

A. *The Metabolism of Iodinated Tyrosine.*

When mono- or diiodotyrosine was incubated with L-amino acid oxidase, each was converted to its respective alpha keto acid and acetic acid, inorganic iodide and unknown compounds. In view of the fact that considerable amounts of the corresponding alpha keto acids are formed from iodinated tyrosines prior to any great increase in inorganic iodide, it may be concluded that iodinated tyrosines were deaminated prior to their deiodination and then subsequent alpha keto acids were deiodinated directly

or indirectly by L-amino acid oxidase. The formation of 3-iodo-4-hydroxyphenylpyruvic acid from diiodotyrosine is attributable to deiodination of 3,5-diiodo-4-hydroxyphenylpyruvic acid formed enzymatically from diiodotyrosine.

However, the conversion of the diiodopyruvic acid to the monoiodopyruvic acid is apparently not the main pathway of its deiodination, because there is no evident increase in the monoiodopyruvic acid during decomposition of the diiodopyruvic acid. Consequently, most of the inorganic iodide would appear to come directly from 3,5-diiodo-4-hydroxyphenylpyruvic acid, probably by splitting of its phenyl ring.

The acetic acids corresponding to the amino acids could be formed by the oxidative decarboxylation of the corresponding alpha keto acids in the presence of hydrogen peroxide, formed during the deamination of the amino acids by L-amino acid oxidase as previously described by some workers (9).

In view of the fact that the degradation of diiodotyrosine by cobra venom requires a longer incubation period than that of monoidotyrosine, it seems likely that the enzymatic action of L-amino acid oxidase upon iodotyrosines is dependent upon the number of substituted iodine atoms in the phenyl ring. However this does not mean that deiodination *in vivo* is effected by L-amino acid oxidase.

Roche *et al.* (13) have suggested that in mammalian tissues there is an enzyme other than L-amino acid oxidase which induces a release of inorganic iodide from the phenyl ring of mono- and diiodotyrosine without a change in the side chain. We have some unpublished data on this point obtained by the incubation of mono- and diiodotyrosine with kidney and liver slices of rats. It seems likely, from these results, that deiodination of the iodotyrosines by these tissues is indeed strikingly different from that observed with cobra venom. In our tissue slice studies deiodination occurred with a greater production of inorganic iodide, less formation of the corresponding alpha keto acids, and there were no peaks indicating formation and decomposition of the corresponding alpha keto acids. Consequently, it may well be, as Roche *et al.* suggest, that the iodotyrosine are deiodinated largely by such a specific iodinase or other enzymes in mammalian tissues. On the other hand a part of their deiodination in the tissues could be effected by L-amino acid oxidase.

B. *The Metabolism of Iodinated Thyronines.*

The decomposition of iodothyronines under the influence of snake venom is quite different in that, in contrast to the iodotyrosines, the iodothyronines do not release inorganic iodide as a metabolite. In addition, the main organic product of the thyronines is the respective acetic acid derivative, whereas the chief metabolite of the iodinated tyrosines in the corresponding alpha keto acid. The unknown compound reported to be a metabolite of triiodothyronine produced by mitochondria of kidney (4, 5) has the

same Rf, using tertiary amyl alcohol saturated with 2N NH_4OH, as TX_3 formed from triiodothyronine by cobra venom in our experiments. On the other hand, we found only one unknown compound (TX_4) in the present experiments in contrast to the studies just cited (4, 5).

The formation of the corresponding acetic acid from iodothyronines by L-amino acid oxidase might suggest that the pyruvic analogues are intermediates in the formation of the acetic acid analogues. TX_3 and TX_4 formed from triiodothyronine and thyroxine respectively might be triiodothyropyruvic acid and tetraiodopyruvic acid but were not identified as such in the present experiment. Actually, Roche *et al.* (2, 3) have already reported the occurrence of triiodothyroacetic acid and pyruvic acid in the kidney of rats treated with radioactive triiodothyronine. Our work could be taken to support their finding and also serve to verify the probable importance of the deamination mechanism of iodothyronine in the tissue, since the kidney is abundantly rich in L-amino acid oxidase. The degradation of triiodothyronine by cobra venom was more marked than that of thyroxine, and this same relationship was evident in the metabolism of mono- and diiodotyrosine under the influence of this enzyme preparation.

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CORTICOSTEROIDS AND TRANSAMINASE ACTIVITY: THE SPECIFICITY OF THE GLUTAMIC-PYRUVIC TRANSAMINASE RESPONSE[1]

FRED ROSEN, NIRA R. ROBERTS, LOUIS E. BUDNICK AND CHARLES A. NICHOL

Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York

ABSTRACT

Marked increases (2 to 5-fold) in glutamic-pyruvic transaminase activity were observed in the livers of rats given cortisol, cortisone or several other corticosteroids subcutaneously for 4 consecutive days. Two biologically inert analogues of cortisol, 11-epicortisol and 9α-methoxycortisol acetate, were essentially inactive in this respect. Treatment with cortisol increased the activity of this transaminase markedly in thymus, liver and pancreas, to a lesser extent in kidney, and to no significant degree in spleen, muscle, heart, diaphragm, adrenal, testis and lung.

The administration of adrenocorticotrophic hormone doubled the activity of glutamic-pyruvic transaminase in liver. Hypophysectomy increased the endogenous level of the transaminase in liver. Injection of cortisol elicited an increase in the activity of this transaminase in the liver of adrenalectomized or hypophysectomized rats which was similar to that observed in intact animals. Growth hormone, testosterone and insulin did not significantly affect liver glutamic-pyruvic transaminase activity nor did they impair the response of this enzyme when given in combination with cortisol. Slight increases in transaminase activity occurred when rats were injected with estradiol or thyroxine; these hormones and progesterone interfered with the transaminase response to cortisol.

THE increased rate of gluconeogenesis and the negative nitrogen balance induced by treatment with corticosteroids is probably accomplished by changes in the activity of certain enzymes concerned with the balance between anabolic and catabolic pathways of metabolism. Recognition of the key position of transamination processes in this regard has resulted in repeated study of these reactions. Recent investigations indicate that one transaminase response related to these metabolic effects is the increased activity of hepatic glutamic-pyruvic transaminase (GPT) following treatment of rats with cortisol (1) or cortisone (2, 3). The response of GPT to treatment with corticosteroids is of particular interest since a closely related transaminase, glutamic-oxaloacetic (GOT), in the

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same tissue did not respond to this treatment (1, 2), leading to the hypothesis that this transaminase (GPT) is rate-limiting in gluconeogenesis (1). Further study in the rat revealed marked increases in the activity of hepatic GPT, but not GOT, in three other conditions associated with enhanced gluconeogenic activity, namely, starvation, alloxan-induced diabetes, and increased levels of dietary protein (4).

This study represents an initial investigation of the specificity of the response of hepatic GPT to corticosteroids and of the effect of other steroids and hormones on this enzyme. Also, the response of this transaminase to cortisol was studied in different tissues of intact animals and in the liver of adrenalectomized and hypophysectomized rats.

METHODS AND MATERIALS

Male rats of the Sprague-Dawley strain weighing from 75–125 gm. were used. The effects of estradiol and progesterone on the GPT activity of liver were studied in rats of both sexes. The animals were fed Purina Laboratory Diet and water *ad libitum*. The adrenalectomized rats were given physiological saline instead of water.

Immature male hypophysectomized rats (85–110 gm.) were obtained from the Charles River Breeding Labs., Wilmington, Mass. Bilateral adrenalectomy was performed under ether anesthesia using a posterior approach. Both the adrenalectomized and hypophysectomized rats received daily injections of cortisol; treatment was begun between the seventh and fourteenth day of the postoperative period. The completeness of adrenalectomy was determined at autopsy, and if residual adrenal tissue was found, the animal was not included in the experiment.

All rats were killed by exsanguination after being stunned by a blow on the head. The tissues were removed rapidly and chilled promptly in crushed ice. Tissues were kept at 0° C until they were homogenized and assayed. Ice-cold distilled water was used for all dilutions. Homogenates of the tissues (1 to 20 dilution) were obtained in the Waring Blendor within 1½ minutes. A Potter-Elvehjem type all-glass homogenizer was used for certain small samples of tissue. The homogenates retained full transaminase activity when stored at −20° C; however, it was necessary to rehomogenize the thawed samples to obtain reproducible transaminase values (4). GPT activity was measured by a procedure of Lowry and co-workers (5). The method is similar in principle to that used by Wroblewski and LaDue (6) except that instead of measuring spectrophotometrically the disappearance of reduced diphosphopyridine nucleotide (DPNH), the DPN formed is determined fluorometrically (7). Protein was measured in tissues by a modification of the Folin procedure (8).

The commercial preparations used were cortisol acetate and cortisone acetate (Merck, Sharpe and Dohme Co.), insulin (E. R. Squibb and Sons), testosterone propionate in oil, estradiol U.S.P. (Diogyn), progesterone (C. Pfizer & Co.) and ACTH (ACTHar gel, Armour Laboratories). Thyroxine was purchased from Nutritional Biochemicals Co. The other hormones were generously supplied by the following sources: growth hormone (F. W. Horner, Ltd.); $\Delta^{1,4}$-pregnadiene-11β,17α,21-triol-3,20-dione (Prednisolone), 6α-methyl-$\Delta^{1,4}$-pregnadiene-11β,17α,21-triol-3,20-dione (Medrol), 9α-fluorocortisol and 9α-fluoro-21-desoxy derivative of Medrol (Upjohn Co.); 11-epicortisol and 9α-methoxycortisol acetate (Squibb Institute for Medical Research). Cortisol, cortisone, testosterone, estradiol and ACTH were injectable preparations. Growth hormone was dissolved in saline and progesterone and thyroxine in propylene glycol.

The corticosteroids and the inactive analogues were dissolved or suspended in propylene glycol and the amounts indicated were injected subcutaneously in a total volume of 0.2 ml.

RESULTS

Effect of Corticosteroids. In previous experiments, it was observed that the administration of large doses of cortisol, cortisone or Prednisone daily for 7 days resulted in striking increases in the activity of GPT of rat liver (1). Five-fold increases in GPT activity were obtained with doses of cortisol which did not cause any decrease in body weight. Several potent corticosteroids were compared with cortisol and cortisone with respect to

TABLE 1. EFFECT OF CORTICOSTEROIDS, INACTIVE ANALOGUES OF CORTISOL, AND CORTICOTROPHIN ON THE HEPATIC GLUTAMIC-PYRUVIC TRANSAMINASE ACTIVITY OF INTACT RATS

Compound	Dose	Liver weights	Glutamic-pyruvic transaminase activity*	
	mg. per day	gm.	per gm. liver	per gm. liver protein
Control†	—	5.45 ±0.60‡	0.995 ±0.30‡	6.66 ±1.9‡
Cortisone acetate	1.0	5.69 ±0.56	4.03 ±0.53	27.0 ±3.9
Cortisol acetate	1.0	4.45 ±0.36	5.70 ±1.7	34.7 ±7.5
9α-Fluorocortisol	0.5	4.28 ±0.19	2.84 ±0.58	18.7 ±2.8
Prednisolone	0.5	4.80 ±0.47	1.74 ±0.41	12.0 ±2.6
Medrol	0.5	4.43 ±0.32	2.92 ±0.20	20.1 ±2.0
9α-Fluoro-21-desoxy derivative of Medrol	0.5	4.71 ±0.49	5.16 ±0.50	34.9 ±3.7
11-Epicortisol	2.0	6.19 ±0.44	1.08 ±0.23	7.52 ±1.7
9α-Methoxycortisol acetate	1.0	5.66 ±0.34	1.29 ±0.33	8.91 ±2.3
	units per day			
Corticotrophin (ACTH)	1	5.07 ±0.17	1.04 ±0.22	6.80 ±1.4
Corticotrophin (ACTH)	2	6.30 ±0.72	0.945 ±0.36	6.12 ±2.2
Corticotrophin (ACTH)	10	7.26 ±1.1	1.48 ±0.24	9.97 ±1.4
Corticotrophin (ACTH)	20	6.02 ±0.83	2.94 ±0.56	18.5 ±2.8

* Millimoles of substrate utilized per hour at 38°.
† Control animals received 0.2 ml. of propylene glycol which was the solvent used for the compounds tested with the exception of the commercial preparations of cortisol acetate and cortisone acetate.
‡ Average ± the standard deviation.

the response of GPT (Table 1). The subcutaneous injection of 1.0 mg. of cortisone or cortisol daily for 4 days resulted in 4- and 5-fold increases, respectively, in GPT activity. Similarly, administration of 4 synthetic corticosteroids caused an increase in hepatic GPT activity; this response, however, was relatively small in view of the comparatively higher potency of these compounds indicated by bioassays.

Inactive analogues of cortisol. Further correlation concerning the specificity of this response was obtained by testing structural analogues of cortisol, 11-epicortisol and 9α-methoxycortisol, which are inactive with respect to the glycogen deposition and anti-inflammatory assays. These compounds were essentially inactive in inducing a GPT response in com-

parison with the effect of other corticosteroids which were tested within the same experiment (Table 1).

Response to adrenocorticotrophic hormone. The subcutaneous injection of 10 U.S.P. units of ACTH twice daily for 7 days more than doubled the GPT activity of liver. A significant increase also occurred when 5 units of ACTH were given twice daily but smaller amounts were without effect (Table 1).

Effect of endocrine ablation. The effect of adrenalectomy or hypophysectomy on GPT activity in the livers of rats treated with cortisol is shown in Table 2. In the immature adrenalectomized rat (100 to 125 gm. in

TABLE 2. EFFECT OF ENDOCRINE ABLATION ON THE RESPONSE OF GLUTAMIC-PYRUVIC TRANSAMINASE TO CORTISOL

Cortisol	Endocrine status	No. rats	Liver weights	GPT activity*
mg. daily ×7			gm.	
0	Intact	30	5.20 ± 1.6†	10.6 ± 2.7†
1.0	Intact	10	4.82 ± 0.84	38.3 ± 4.4
2.5	Intact	15	8.90 ± 2.4	51.6 ± 5.0
5.0	Intact	10	7.65 ± 0.77	57.6 ± 4.8
0	Adrenalectomized	5	8.90 ± 1.5	14.7 ± 8.2
1.0	Adrenalectomized	5	9.50 ± 0.82	23.3 ± 4.4
2.5	Adrenalectomized	5	9.97 ± 0.32	44.9 ± 3.4
5.0	Adrenalectomized	5	10.68 ± 0.89	57.7 ± 8.1
0	Hypophysectomized	5	3.99 ± 0.58	18.4 ± 2.1
1.0	Hypophysectomized	5	5.54 ± 0.48	65.5 ± 4.8
2.5	Hypophysectomized	5	4.70 ± 0.25	69.0 ± 3.4
5.0	Hypophysectomized	5	3.84 ± 0.80	75.6 ± 3.7

* Millimoles substrate utilized per gm. protein per hour at 38° C.
† Average ± the standard deviation.

weight), the response to cortisol was similar to that observed in the intact animal. In other studies, the GPT activity in liver four weeks after adrenalectomy was considerably lower than in intact animals.

The values for GPT in liver of untreated hypophysectomized rats were higher than those found in normal rat liver (Table 2). Treatment with 1.0 mg. of cortisol daily produced a higher level of GPT activity in the liver of the hypophysectomized rats than was observed in either the adrenalectomized or intact rats within the same experiment even at higher dosage (Table 2). Since the response of the hypophysectomized rats was almost maximal at the 1.0 mg. dose level, study of this response at lower dosage is needed to evaluate the degree of sensitivity of the hypophysectomized rat.

Effect of hormones other than corticosteroids. Other hormones were tested to determine whether they influenced the normal activity of GPT in liver, or whether they might modify the transaminase response obtained with cortisol when administered simultaneously. Treatment with growth hormone, testosterone and insulin, in the dosages used, did not significantly affect normal liver transaminase activity nor did they impair the response

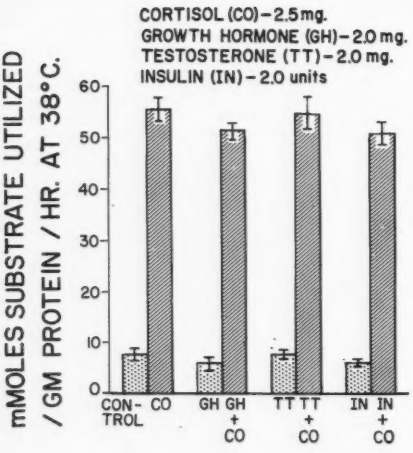

FIG. 1. The response of glutamic-pyruvic transaminase to cortisol given in combination with other hormones. The doses indicated were injected/rat/day for 7 days. Five animals were used in each group. The vertical columns indicate the average values and the standard deviation is shown by the closed bars.

of this transaminase when given in combination with cortisol (Fig. 1). The effects observed with growth hormone are in agreement with the studies of Beaton *et al.* (9) who found that treatment with this hormone does not affect the level of this transaminase in young rats.

Significant increases in hepatic GPT activity were obtained in rats injected with large amounts of estradiol and thyroxine daily for one week (Fig. 2). However, this response was much smaller than that observed with cortisol. When injected simultaneously with cortisol these hormones, as well as progesterone, appeared to interfere with the response of this enzyme to cortisol and resulted in lower activity of GPT as compared to the activity found with cortisol treatment alone. The inhibitory effect of both progesterone and estradiol on the GPT response elicited by cortisol

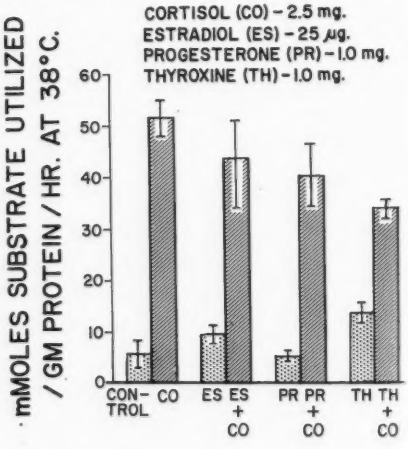

FIG. 2. The effect of other hormones on the activity of glutamic-pyruvic transaminase when given in combination with cortisol. The doses indicated were injected /rat/day for 7 days. Five animals were used in each group. The vertical columns indicate the average values and the standard deviation is shown by the closed bars.

was noted in both male and female animals. The values for estradiol do not appear to be significant in this experiment due to the large standard deviation (Fig. 2). In another experiment, however, the GPT activity in liver following treatment with estradiol (25 μg.) and cortisol (2.5 mg.) was 39.9 ± 0.6 millimoles of substrate utilized per gm. of protein, whereas the value obtained with cortisol (2.5 mg.) alone was 55.7 ± 2.3 millimoles of substrate utilized per gm. of protein.

TABLE 3. EFFECT OF CORTISOL ON GLUTAMIC-PYRUVIC TRANSAMINASE ACTIVITY IN VARIOUS TISSUES OF THE RAT*

Tissue	Organ Weights, gm.		Glutamic-pyruvic transaminase activity†		
	Control	5.0 mg. Cortisol‡	Control	5.0 mg. Cortisol‡	Treated/ Control
Thymus	0.39 ±0.04§	0.034 ±0.008§	0.34 ±0.33§	5.5 ±1.95§	16.2
Liver	6.29 ±0.76	7.09 ±0.47	14.4 ±4.7	62.2 ±4.5	4.3
Pancreas	0.35 ±0.09	0.33 ±0.07	5.1 ±1.1	13.3 ±0.50	2.6
Kidney	0.65 ±0.03	0.60 ±0.01	0.66 ±0.12	1.1 ±0.22	1.7
Testes	0.69 ±0.08	0.70 ±0.08	0.51 ±0.04	0.65 ±0.07	1.3
Spleen	0.38 ±0.05	0.12 ±0.01	0.19 ±0.02	0.22 ±0.05	1.2
Muscle	0.87‖	0.63‖	3.4 ±0.32	4.0 ±0.61	1.2
Diaphragm	0.23‖	0.30‖	7.5 ±0.86	8.0 ±2.3	1.1
Adrenals	0.024 ±0.002	0.010 ±0.001	1.1 ±0.14	1.0 ±0.30	0.92
Lung	0.28 ±0.09	0.20 ±0.08	2.3 ±0.16	1.8 ±0.27	0.78
Heart	0.51 ±0.03	0.47 ±0.02	2.3 ±0.20	1.7 ±0.37	0.72

* 5 to 9 rats per group.
† Millimoles of substrate utilized per gm. protein per hr. at 38°.
‡ Amount injected per day for 7 days.
§ Average ± the standard deviation.
‖ Average weights; no attempt was made to remove the entire gastrocnemius or diaphragm muscles.

Effect of cortisol treatment on GPT activity in different tissues. The levels of GPT in various tissues of the rat, as well as the values found following treatment with cortisol are shown in Table 3. It is of interest to note the wide variation in GPT activity found in the different tissues which were studied. The level of enzyme activity was particularly low in thymus gland, as well as in kidney, testes and spleen. The highest level of activity occurred in liver, followed in decreasing order by diaphragm, pancreas, skeletal muscle, heart muscle, lung and adrenals.

In rats treated with 5 mg. of cortisol daily for one week increased activity of GPT occurred in thymus, liver and pancreas, to a lesser extent in kidney, and to no significant degree in seven other tissues. The greatest relative increase in activity occurred in thymus gland. The GPT activity of thymus in rats treated with cortisol was increased more than 16-fold on the basis of the protein content of the tissue. This treatment was associated with a 95% reduction in the size of this gland.

DISCUSSION

Among the steroids which have been studied to date, without exception all of the compounds known to have corticosteroid activity have increased

the activity of glutamic-pyruvic transaminase in the liver of treated rats. Several steroids, closely related in structure but without corticosteroid activity, did not increase the activity of this transaminase in liver. The biologically inactive isomer of cortisol, 11-epicortisol, did not elicit a GPT response when administered to rats; similarly, 9α-methoxycortisol was inactive. Furthermore, treatment with desoxycorticosterone acetate actually lowered the activity of this transaminase in liver to 50% of the normal value (4). The consistent response of GPT to treatment with corticosteroids, but not to treatment with related steroids which are inactive in this respect, indicates a highly specific association of the response of this transaminase with corticosteroid activity.

A dose response curve of adequate slope to serve as a basis for a bioassay was obtained by the injection of cortisol for four days followed by assay of the GPT activity of liver on the 5th day (4). When a number of the variables which can influence such a bioassay have been defined, it will be possible to compare the relative potencies of different corticosteroids by this enzyme response with the values obtained by other biological tests.

It should be noted that this transaminase response occurs *in vivo* and that maximum increase in enzyme activity occurs after treatment for several days. Such a response may be associated with the secondary adaptation to stress concerned with the utilization of tissue proteins. The increase in transaminase activity may involve the synthesis of new enzyme protein (4). In this case, special requirements for a response in any *in vitro* system would be expected. In initial trials, no change in GPT activity has resulted from the addition of cortisol to homogenate and liver slice preparations. The activity of this enzyme is being studied in suspensions of cells sensitive to cortisol.

The administration of adrenocorticotrophic hormone produced a twofold increase in GPT activity of liver. ACTH did not result in increases as great as those observed following treatment with large doses of cortisol or other corticosteroids. It is probable that the increase in GPT activity in this case is limited by the rapid metabolic inactivation of ACTH (10) as well as by the amounts of corticosterone released from the adrenal glands. A greater increase in this enzyme might have been obtained by injecting ACTH at more frequent intervals, by prolonging the period of its administration, or by injecting large amounts of a highly purified corticotrophin preparation. Doses much larger than used here were necessary to produce a ketogenic response (11) or glycosuria (12) in normal rats.

The adrenalectomized rat appears to be somewhat less sensitive than the intact animal with respect to the magnitude of the GPT response noted following treatment with 1.0 mg. amounts of cortisol (Table 1). It was found previously that significant stimulation of GPT activity occurred in adrenalectomized rats maintained on a high protein diet for one week; this stimulation was less than that observed in normal rats under the same

dietary conditions (13). The finding that high protein *per se* results in increased GPT activity in the adrenalectomized rat indicates that this effect in the intact animal is probably conditioned directly by the level of dietary protein as well as by any stress reaction mediated by the adrenal gland. The response of this transaminase to different levels of dietary protein requires further consideration of the possibility that a substrate-induced enzymatic adaptation is involved.

In untreated hypophysectomized rats, the endogenous GPT activity of liver was higher than the activity routinely found in normal animals. Several investigators (14, 15, 16) have reported an abnormally high activity of glutamic-oxaloacetic transaminase in the liver and muscle of hypophysectomized rats. Treatment of hypophysectomized rats with 1 mg. of cortisol daily for 7 days produced a much greater increase in hepatic GPT activity than in normal rats injected with the same amount of cortisol (Table 2). In this respect, an important consideration may be that the removal of the pituitary gland would abolish the secretion of trophic hormones (gonadotrophins, thyrotrophin, etc.) which would lead to lower levels of circulating estrogen, progesterone and thyroxine. Since these hormones have been found to decrease the response obtained with cortisol, any reduction in their levels in the blood of hypophysectomized rats may be related to the increased sensitivity of the GPT response observed in such animals following treatment with cortisol.

A marked increase (16-fold) in GPT activity was observed in thymus tissue following daily treatment with cortisol for one week. The specificity of this response is demonstrated by the slight increase (less than 20%) in glutamic-oxaloacetic transaminase activity observed under similar conditions. These data also indicate that the rise in GPT activity is not due to the concentration of this enzyme in residual thymus tissue. Blecher and White (17) found that cortisol (5×10^{-4} *M*) added *in vitro* to suspensions of thymic lymphocytes decreased (38–48%) the activity of GOT when the cells were obtained from pre-pubertal rats and increased (38–49%) the activity of this enzyme in the preparations from post-pubertal animals.

In an extension of this investigation, these transaminase enzymes were studied in tumors which are sensitive to cortisol as indicated by suppression of growth. An 8- to 12-fold rise in the GPT activity of Walker carcinoma 256 was noted following treatment with cortisol (2.5 mg. per rat daily for 7 days) whereas the activity of glutamic-oxaloacetic transaminase was not significantly altered (18). The correlation between the activity of these transaminase enzymes and the response of tissues sensitive to corticosteroids deserves detailed study.

In this study, treatment with testosterone, insulin and growth hormone did not alter normal liver GPT activity or affect the response of this enzyme when tested in combination with cortisol. Growth hormone has been noted to reduce the GPT activity in liver of adult rats but not in pre-

pubertal animals (9). The activity of GPT in liver was elevated significantly by the administration of estradiol or thyroxine and a similar effect was associated with hypophysectomy. It is of particular interest that this transaminase response to corticosteroids is suppressed by treatment with estradiol, progesterone and thyroxine. These results indicate that the response of GPT reflects some of the complex interrelationships that exist between corticosteroids and other hormones.

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ESTROGEN ANTAGONISMS: THE EFFECTS OF A SERIES OF RELATIVES OF 19-NORTESTOSTERONE ON ESTRONE-INDUCED UTERINE GROWTH

RICHARD A. EDGREN, DAVID W. CALHOUN, RICHARD L. ELTON AND FRANK B. COLTON

Divisions of Biological and Chemical Research, G. D. Searle and Co., Chicago, Illinois

ABSTRACT

A series of 29 compounds related to 19-nortestosterone has been studied for ability to restrict the uterine growth stimulated by 0.3 μg. of estrone. Potency (relative to progesterone) in the 19-nortestosterone series is related to the length of the side chain at 17, increasing in straight-chain forms to a peak at the ethyl (1,250% of progesterone), and then decreasing to the inactive butyl. Branching, ethyl to isopropyl (2,200%) and allyl (160%) to 1-methallyl (400%) and 2-methallyl (680%), is accompanied by a rough doubling of potency. Unsaturation affects potency: the vinyl (460%) and allyl are less potent than the ethyl and propyl (100–400%), whereas the ethynyl (800%) and propynyl derivatives (550%) are more active than their monounsaturated forms. The 5(10) dehydro isomers (estreneolones), as well as the dihydro-19-nortestosterones are much less active than the first series. 17α-Ethyl-17-hydroxy-5(10)-estren-3-one (20%) and the propyl derivative (15%) are active. 17β-Hydroxy-5(10)-estren-3-one and its 17α-methyl and ethynyl derivatives are inactive. Of the dihydro-19-nortestosterones only the methyl (35%), ethyl (30%) and isopropyl (30%) are distinctly active, and these only when the hydrogens at 5 and 10 are in the β position; 5α, 10α and 5α, 10β isomers were essentially inactive where tested, as were the β-cis forms of dihydro-19-nortestosterone and the three 17-propyl derivatives. These activities are discussed in relation to the other biological properties of these substances: progestational, estrogenic, androgen-anabolic.

THE biological activities of compounds related to 19-nortestosterone have received extensive investigation over the past several years. The actions of these materials as estrogen antagonists have been subjected to study: Payne, Hellbaum and Owens (1) showed that 17-ethyl-19-nortestosterone antagonized the effects of estrogens on the ovary and uterus of hypophysectomized rats. Edgren and Calhoun (2) compared the potencies of 17-ethyl-19-nortestosterone, testosterone propionate and progesterone as antagonists of estrone-induced uterine growth, and the first two of these three compounds were studied for their effects on estrone-induced changes in vaginal cytology (3). Bowers, Ringold and Dorfman (4) synthesized a series of dihydro-19-nortestosterones; they report that these are potent estrogen antagonists, citing dihydro-19-nortestosterone and

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17-methyl-dihydro-19-nortestosterone as examples. Sturtevant (5) reported preliminary data from our laboratory that implicated 17-propyl-dihydro-19-nortestosterone as an inhibitor of estrone-induced uterine growth. Recently, Edgren (6) showed that 17-ethynyl-19-nortestosterone was a potent antagonist, whereas the $\Delta^{5(10)}$ isomer was inactive in the same test. Elton and Edgren (7) implicated the 17-(2-methallyl) derivative.

TABLE 1. POTENCIES OF VARIOUS RELATIVES OF 19-NORTESTOSTERONE AS INHIBITORS OF THE UTERINE GROWTH INDUCED IN INTACT, IMMATURE MICE BY 0.3 μG. OF ESTRONE. ALL POTENCIES ARE RELATIVE TO PROGESTERONE, WHICH WAS ASSIGNED A POTENCY OF 100%, AND WERE EVALUATED AT THE DOSE THAT WAS ESTIMATED TO PRODUCE A DEPRESSION OF GROWTH RESPONSE EQUIVALENT TO 50% OF THAT GROWTH EXPECTED FROM THE ESTRONE ALONE. UNDER N THE PARENTHETICAL NUMBER INDICATES THE NUMBER OF TESTS IN WHICH A GIVEN COMPOUND WAS EVALUATED; THE NUMBER IN FRONT OF THE PARENTHESES INDICATES THE TOTAL NUMBER OF GROUPS OF 8–10 MICE EMPLOYED IN THE POTENCY ESTIMATION

17(α)-Substituent	4-dehydro		5(10)dehydro		4,5-dihydro	
	N	Potency	N	Potency	N	Potency
Progesterone	13(4)	100				
19-nortestosterone "derivatives"						
H	13(2)	40	8(2)	Inactive	9(1)	Inactive (Trans)
					5(1)	Inactive (β-cis)
Methyl	18(3)	880	20(3)	Inactive	14(3)	35 (β-cis)
Ethyl	32(6)	1250	14(3)	20	5(1)	Inactive (Trans)
					12(3)	30 (β-cis)
					5(1)	Inactive (α-cis)
Vinyl	10(2)	460				
Ethynyl	9(2)	800	16(3)	Inactive	14(2)	Inactive (Trans)
Isopropyl	9(2)	2200			17(4)	Inactive (Trans)
					14(3)	30 (β-cis)
n-Propyl	19(4)	100–400	14(3)	15	9(2)	Inactive (Trans)
					19(4)	Inactive (β-cis)
					5(1)	Inactive (α-cis)
Allyl	9(2)	160				
Propynyl	5(1)	550				
n-Butyl	16(2)	Inactive				
1-Methallyl	15(2)	400				
2-Methallyl	26(4)	680				

A long series of these substances has now been studied for progestational activity and effects on estrone-induced uterine growth. Compounds employed and numbers of animals used are summarized in Table 1.

MATERIALS AND METHODS

Estrogen Antagonisms. Our methods have been described in detail in a prior communication (2). Briefly, intact, immature mice were treated with 0.3 μg. of estrone, which

was given in three equal subcutaneous injections over three successive days. Compounds to be tested as estrogen antagonists were mixed with the estrone; both estrogen and putative antagonist were administered in a single injection of 0.1 ml. of oil solution each day. At autopsy, 24 hours after the third injection, the uteri were removed, cleaned of adherent tissues, scored and blotted to remove contained fluid, and weighed wet on a suitable torsion balance.

For convenience in making quantitative comparison among blockers, uterine weight was converted to a percentage blocking scale. According to this scale, a weight equal to that expected from estrone alone would receive the value, 0, while the smaller weight expected following oil injections would receive the value, 100. The average uterine weight following oil in our experiments was consistently about 10 mg., corresponding to 100 on this scale. We found that the uterine weights of estrone-injected animals averaged 35 mg., but that this varied considerably from one experiment to another. Furthermore, these fluctuations were positively correlated with fluctuations of concurrent treated group averages. Thus on the average a uterine weight of 35 mg. received the scale value, 0. However, in any one experiment the zero point might correspond to a higher or lower uterine weight, depending on the simultaneous estrone standard. An appropriate correction for deviations of the estrone group uterine weight from 35 mg. appeared to be 0.25 mg. for each milligram deviation of the estrone standard. A similar adjustment for variations in the oil injected control groups was found unnecessary.

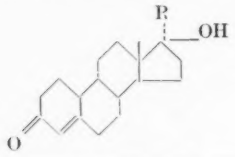
Progestational Activity. Progestational potencies were obtained from a subcutaneous Clauberg test. Intact, immature, female rabbits that weighed about 1 kg. were primed for 6 days with 5 μg. of estradiol-17β per day. Starting on the day following the last priming injection they received the test compound daily for 5 days. On the day after the final injection the rabbits were sacrificed and a segment of uterus was removed for histological analysis. Upon microscopic examination, the degree of glandular proliferation of the endometrium was rated on a +1 to +4 basis. A+1 response indicated a uterus characterized by a lack of glandular development, whereas a +4 rating indicated maximal proliferation of the glands. For purposes of this discussion potency estimations were based upon comparisons among the minimal doses that produced +3 responses.

RESULTS AND DISCUSSION

The estrogen-antagonism and progestational activities of the materials discussed below are summarized in Tables 1–4. In order to avoid any detailed consideration of the nature of the interactions between these compounds and estrone, we have arbitrarily chosen to compare relative blocking effectiveness at a single level of response. This oversimplification brings out the prominent features of structure-activity relationships, but introduces certain apparently anomalous evaluations that must be noted briefly. Previous data (8), employing progesterone and testosterone propionate, strongly suggested that the former was a competitive inhibitor of estrone-induced uterine growth, whereas the latter fitted a competition model imperfectly. Perhaps the 19-nortestosterones compete with estrone for substrate or point of attachment, possibly in the uterus. If these materials are actually competitors, then the inhibitory response would be opposed by any growth produced by the antagonist itself. In evaluating potencies of estrogen antagonists, we have compared doses producing a median response of 50, which corresponds to an average uterine weight of

22.5 mg., in combination with estrone. Antagonists with metrotropic action thus will not be distinguishable from less active antagonists that lack such action. If an antagonist produced uterine responses of 22.5 mg., it would be classed as inactive. Thus testosterone and testosterone propionate are considered as "inactive," according to this evaluation, and other compounds with inherent metrotropic effects are less potent than when estimated by other methods (2).

TABLE 2. COMPARISON OF ESTROGEN-ANTAGONISTIC AND PROGESTATIONAL ACTIVITIES OF A SERIES OF 17 SUBSTITUTED 19-NORTESTOSTERONES. THE PROGESTATIONAL POTENCIES WERE OBTAINED FROM A CLAUBERG TEST



17-Substitutent (R)	Antagonism potency*	Progestational potency*
H	40	Inactive
Methyl	880	30
Ethyl	1250	750
Vinyl	460	150
Ethynyl	800	50
Isopropyl	2200	2000**
n-Propyl	100–400	300
Allyl	160	500
Propynyl	500	500
n-Butyl	Inactive	100
1-Methallyl	400	500
2-Methallyl	680	1200

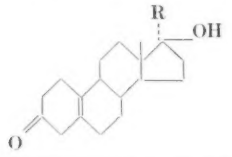
* Progesterone = 100%.
** Preliminary data.

19-Nortestosterones (Tables 1, 2). With the exception of the butyl, all of the 19-nortestosterone derivatives were active estrogen antagonists. The potencies of the materials in this test would appear to correlate with the length of the chain at 17. Among the straight-chain compounds, potency increases from 19-nortestosterone itself, the least potent of the active compounds, to a peak at the ethyl. The propyl is less active than the ethyl and the butyl appears to be devoid of any effect. In this series, unsaturation and branching of the side chain also appear to influence potency. Unsaturation, ethyl to vinyl, and propyl to allyl, appears to produce a decrease in potency, whereas the acetylenic forms, ethynyl and propynyl are more active than the compounds with monounsaturated side chains. The introduction of a branch in the ethyl side chain to form the isopropyl is

accompanied by a rough doubling of potency; similarly the 1-methallyl is about twice as potent as the allyl. The 2-methallyl is more potent than the 1-methallyl, suggesting that the distance from the point of branching to the nucleus may be important.

Preliminary data on these materials suggested that our antagonism assay amounted to an alternative method of testing progestins. In general our data support such an hypothesis. However, the absence of a direct

TABLE 3. COMPARISON OF ESTROGEN-ANTAGONISTIC, PROGESTATIONAL AND ESTROGENIC ACTIVITIES OF A SERIES OF 17α-SUBSTITUTED 17β-HYDROXY-5(10)-ESTREN-3-ONES (ESTRENEOLONES). ESTROGENIC DATA FROM UTERINE GROWTH STUDIES ON INTACT, IMMATURE MICE (RUBIN TEST)



17α-Substituent (R)	Antagonist potency*	Progestational potency*	Estrogenic potency**
H	Inactive	Inactive***	1
Methyl	Inactive	Inactive***	1****
Ethyl	20	50	Inactive
Ethynyl	Inactive	Inactive***	7
n-Propyl	15	50	Inactive

* Progesterone = 100%.
** Estrone = 100%.
*** Active, but less so than standard set here.
**** Impeded.

glandular effect of 19-nortestosterone, an antagonist, and, conversely, the absence of estrogen antagonistic action of the butyl, which is still an active progestin, suggest that these activities are separated. With respect to certain compounds, quantitative evaluations demonstrate a marked lack of parallelism between the two tests.

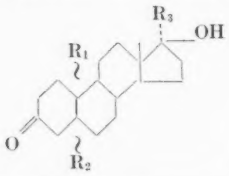
There would appear to be no correlation between estrogen antagonistic action and androgen-anabolic activities in this series, since two of our highly active materials are reported as essentially inactive as androgenic-myotropic agents, namely the ethynyl and 2-methallyl (9, 10). The ethynyl has been reported as slightly estrogenic by Pincus, *et al.*, (11) and various other workers (9, 12, 13, 14); however, in our hands highly purified samples of this material have appeared nonestrogenic (6). Saunders' evaluations (9, 12) were based upon seminal vesicle growth (personal communication), which since the slope of the dose-response curve was shallow, appeared to be of the estrogenic type.

Differences between potencies cited here and those cited in previous publications from this laboratory (2, 7) are, at least in part, explicable on the basis of the change in statistical approach. In addition, since the experi-

ments that were reported in the previous paper were completed (2), it has been necessary for us to change mouse strain. Our earlier work was carried out using Stokeley-Peterson mice and the studies reported here were based on Rockland All Purpose mice; it seems not unlikely that differences in uterine sensitivity are involved (15).

Estreneolones (Tables 1, 3). The $\Delta^{5,10)}$ isomers of these 19-nortestosterones can be classified as 17α-substituted estreneolones. They show definite

TABLE 4. COMPARISON OF ESTROGEN-ANTAGONISTIC AND PROGESTATIONAL ACTIVITIES OF A SERIES OF 17-SUBSTITUTED DIHYDRO-19-NORTESTOSTERONES



17-Substitutent (R_3)	Hydrogen		Antagonist potency*	Progestational potency*
	10(R_1)	5(R_2)		
H	β	α	Inactive**	Inactive**
	β	β	Inactive	Inactive
Methyl	β	β	35	10
Ethyl	β	α	Inactive	100
	β	β	30	50
	α	α	Inactive	
Ethynyl	β	α	Inactive	Inactive
Isopropyl	β	α	Inactive	
	β	β	30	Active
n-Propyl	β	α	Inactive	Inactive
	β	β	Inactive	Inactive
	α	α	Inactive	Inactive

* Progesterone = 100%.
** Active, but less so than standards set here.

estrogenic as well as progestational, and estrogen antagonistic activities. None of these materials is particularly potent in any of these areas, but various interesting correlations exist among the three types of activities. In our experience with this testing procedure, no material that has estrogenic activity itself, such as estriol or epi-estriol, has been an antagonist. Although this has proved true for all studied materials, it has only been described for 17α-ethynyl-17-hydroxy-5(10)-estren-3-one (6). Among the estreneolones in the present series, 17β-hydroxy-5(10)-estren-3-one and its 17α-methyl and 17α-ethynyl derivatives are estrogenic, whereas the ethyl and propyl derivatives are inactive. Only these latter two materials were antagonists; in addition they showed much more progestational ac-

tivity than the other compounds. It would appear from the few estreneolones investigated that progestational and estrogen antagonistic effects are directly correlated, whereas estrogenic activities are demonstrated only by the materials that show minimal progestational potency on subcutaneous administration.

Dihydro-19-nortestosterones (Tables 1, 4). Although none of these compounds is particularly potent, the series again shows an interesting pattern of activities. Only three of the materials fall within the definition of activity employed here. A fourth is actually active, but not within the limitations we have set. Of the patently active compounds in this series all have the 5 and 10 hydrogens in the β position; the β-cis isomers of the methyl, ethyl and isopropyl are all weakly active. In addition to these three materials the β-cis isomer of the propyl was tested and found to be essentially devoid of activity, as were the α-cis and 5α, 10β isomers of all compounds. Qualitatively the trans isomer of dihydro-19-nortestosterone shows slight activity, although, at dose levels tested, it did not produce a 50% depression of uterine response. We are unable to confirm Bowers, Ringold and Dorfman (4) or Ringold (16) who state that it is 8–10 times more potent than 19-nortestosterone; in our test it is at most equal to 19-nortestosterone. The absence of activity of the β-cis isomer of the propyl requires retraction of Sturtevant's (5) statement to the contrary, which was based upon preliminary data that we have been unable to confirm.

Clauberg studies on this series have not yet progressed sufficiently to give quantitative evaluations for all compounds. Each of the active estrogen antagonists is also a progestational agent. Of the materials that have been tested there is a correlation of activities with respect to all compounds except the trans isomer of the ethyl derivative, which shows some progestational activity, but is not an estrone antagonist.

General Remarks. There appears to be some correlation between the 19-nortestosterones and the dihydro-19-nortestosterones. Only the β-cis isomers of the dihydro-19-nortestosterone series are obviously active, and their activities correspond to the greatest potencies in the nortestosterone group. Thus the methyl, ethyl and isopropyl derivatives of 19-nortestosterone are active in this series and all are over 8 times more potent antagonists than progesterone. One might predict that the β-cis isomers of the 17-ethynyl and perhaps the 2-methallyl would be active.

Correlation of activities between the 19-nortestosterones and the estreneolones are not obvious, if they exist at all. The high activity of 17-ethyl-19-nortestosterone is reflected by activity in the ethyl estreneolone derivative, whereas the only other active estreneolone is the propyl, which is characterized by lowered potency in the 19-nortestosterone series. It would appear that estrogenicity and isomeric specificities are confusing the correspondence here.

Huggins, Mainzer and Briziarelli (17) have discussed certain of these materials with reference to their effects on growth of a class one mammary fibroadenoma. The shorter side chains, hydrogen to ethyl, of 19-nortestosterone show increasing activity that is correlated with increasing length of the chain. Beyond the ethyl, activity would appear to be lost; both the propyl, among the saturated side chains, and the ethynyl actually stimulate growth of the tumor. Huggins, *et al.*, have only tested two compounds of the estreneolone series. The ethyl, an active antagonist in our test, was also an inhibitor of tumor growth, and the ethynyl was essentially inactive in both tests. There would appear to be some relationship between these two types of activity, although the nature of the relationship remains obscure.

Acknowledgments

We are particularly indebted to Dr. F. J. Saunders of the Division of Biological Research, G. D. Searle and Co., for allowing us access to his unpublished data on these compounds. Although most of the reported steroids were prepared by one of us (FBC), Drs. Raymond E. Counsell and Raphael Pappo and Messers Leonard N. Nysted and Richard Ray were extremely helpful in synthesizing various of these compounds. The technical assistance of Grace Perkins, DeAnn Peterson, Jacquelyne Steiner, Mary Anne Ryan, Mary Marquez, Virginia Schutte, Brian Olson, Barbara Kitch, Juliette Sleeper, Sally Baker, Christine Motter and Nira Davy is gratefully noted.

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BIOCHEMISTRY OF AMPHIBIAN METAMORPHOSIS: EFFECT OF TRIIODOTHYRONINE, THYROXIN, AND DINITROPHENOL ON THE RESPIRATION OF THE TADPOLE[1,2]

EMMA JO CASSON LEWIS AND EARL FRIEDEN

Department of Chemistry, Florida State University, Tallahassee, Florida

ABSTRACT

A simple method involving the Warburg apparatus has been used for the study of the oxygen consumption of small and moderate sized tadpoles. Significant increases in the respiration of tadpoles were observed after small doses of 3,5,3′-triiodothyronine, regardless of the unit of tissue used for the calculation. Thyroxin also increased tadpole oxygen consumption. However, it was not possible to establish whether or not spontaneous metamorphosis is accompanied by an increase in respiration. The tadpole gave a calorigenic response to 2,4-dinitrophenol whether administered by injection or by immersion. No significant increase was noted in succinoxidase activity after 3,5,3′-triiodothyronine treatment. The possibility of the greater importance of alternative paths of oxidative metabolism in the tadpole is suggested.

THAT mammalian respiration is stimulated by the thyroid hormone is widely accepted (1). Increase in certain respiratory enzymes as well as many other enzymes after thyroid treatment has often been noted (2). It has generally been assumed that the process of differentiation in amphibia, which is partially under thyroid control, would likewise be accompanied by an increase in oxygen consumption. Yet there is not universal agreement on this point.

In his book, Needham (3) considered the matter settled and noted that the early evidence supported the view "that oxygen consumption of the larva rises both absolutely and relatively on metamorphosis, whether naturally or provoked by thyroxin." This position has not been accepted by Etkin (4). As recently as 1955, Etkin (4) wrote that "it is doubtful whether the thyroid does exert a metabolism-increasing effect upon amphibian larvae." Etkin (5) has contended that the increase in oxygen con-

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[2] The assistance of Mrs. Alice Caldwell in some of the analyses is gratefully acknowledged.

sumption per unit weight that had been reported during thyroid-induced amphibian-metamorphosis did not actually represent an increase in metabolic rate, since it resulted from the loss in body matter rather than an increase in oxygen consumption per individual or per unit dry weight.

Etkin (4) further raised the question as to whether or not a metabolic effect is of causal significance in metamorphosis. In this viewpoint he was joined by Needham (3) who agreed that the rise in metabolism is neither the cause of, nor a necessary condition for, metamorphosis. But these conclusions were based upon different and disputable data. On the other hand, Barker (1), in his comprehensive review of the thyroid hormone, concluded that the thyroid gland may be concerned exclusively with energy metabolism and may indirectly be involved in making the necessary energy available for many specialized processes, including metamorphosis and differentiation.

In a survey of some of the respiratory enzymes of the tadpole during normal and induced metamorphosis, we noted the absence of a significant increase in certain key oxidative enzymes such as succinoxidase (6). This was unexpected in view of the good correlation between oxygen consumption and succinoxidase activities for rat tissues noted by Barker (7). We were thus prompted to reexamine the question of alterations of the oxygen consumption during the transition of the tadpole to the frog. A simple technique for measuring oxygen consumption of tadpoles using centerwell-less Warburg vessels as respiration chambers was used. Because of the lack of restriction on the activity of the metamorphosed tadpole, this method has not yielded the answer to the question of oxygen consumption during spontaneous metamorphosis. However, significant increases in respiration in response to Trit[3] and Tx[3] have been obtained, regardless of the unit of tissue used for the calculation. Méhes and Berde (8) have reported an increase in tadpole respiration after immersion in 1:10,000,000 Tx. Méhes and Berde (8) also reported that immersion in DNP[3] had no effect on the metabolic rate of the tadpole. However, we have observed an impressive calorigenic response of the tadpole to DNP.

EXPERIMENTAL

Tadpoles.—Several thousand *Rana grylio* tadpoles were collected in June, 1956, from a local pond. This species, commonly known as the southern bull frog, can be maintained under laboratory conditions for many months without rapid metamorphic development. *Rana heckscheri* and *Bufo americanus* tadpoles were also collected from local sources. *Rana catesbeiana* tadpoles were purchased from the Lemberger Company, Oshkosh, Wisconsin.

Oxygen Consumption.—R. grylio tadpoles of 1.0 and 1.5 gm., with a hind limb to tail ratio of less than 0.20, were used, except where noted, in all respiration studies. For most

[3] The following abbreviations are used: Trit—L-3,5,3'-triiodothyronine; Tx—L-thyroxin; DNP—2,4-dinitrophenol.

experiments there were selected two groups of eight tadpoles, each of whose respiration differed from an average by no more than 10%. The animals were maintained in individual stacking dishes and were not fed during respiration experiments. They were measured and weighed prior to each experiment. They were then placed in large Warburg vessels without centerwell and enough tap water added to bring the final volume to 5.0 ml. The side arm was used for the sodium hydroxide trap and included an appropriate filter paper accordion. The Warburg vessels were equilibrated for 20 minutes and oxygen consumption determined for the next 60 minutes. The temperature of the bath was 23.0° C, which closely approximated the average room temperature. At the termination of the experiment the tadpoles were removed from the Warburg vessels and returned to their individual dishes. The treatment described here did not appear to injure the animals. The results were averaged and the calculated standard deviations are shown by the vertical bars on the respective graphs. Although it was not known to us when first used in this laboratory, this technique is apparently similar to that used by Méhes and Berde (8).

Succinoxidase Measurement.—In several experiments, following the last oxygen consumption measurement, ice cold distilled water homogenates containing 10% tadpole tissue by weight were prepared. The succinoxidase was measured essentially as described by Schneider and Potter (9). The succinoxidase system has been studied earlier in the tadpole (6) and appears to be sufficiently similar to the rat enzyme to warrant similar treatment. In other experiments, tadpole organs were removed and 10% homogenates prepared and used immediately for succinoxidase determination.

Other Measurements and Preparations.—Tadpoles were rapidly weighed on a triple-beam balance in a semi-moist state by allowing excess water to drain off. A semi-moist condition must be maintained in order to avoid permanent damage to the tadpoles. Repeated weighings indicated a variation of less than 10%. The tails were measured at various intervals and the per cent decrease in tail length calculated.

When treated, except as otherwise indicated, tadpoles were injected intraperitoneally using a 27 gauge needle, with the amount of drug[4] indicated in the various figures. Controls were injected with saline.

Tadpole homogenates were prepared in the cold and samples immediately removed for succinoxidase measurements. The homogenates were frozen and rehomogenized when necessary for subsequent dry weight determination and nitrogen analysis. Aliquots were analyzed for nitrogen, after an acid digestion with the aid of H_2O_2, by direct nesslerization.

RESULTS AND DISCUSSION

Effect of Trit on the Oxygen Consumption of Tadpoles.—The effect of two different amounts of Trit are indicated in Figures 1 and 2. In these and most subsequent figures, the data are expressed in terms of microliters oxygen per hour per animal as well as per gram of wet weight. Data for the change in weight are included. Finally, to indicate the morphological response to Trit or Tx, the per cent decrease in tail length is also indicated.

In Figure 1, a minimal dose of Trit was used representing only 10^{-9} moles Trit per gram of tadpole. Only a small decrease in weight or tail length is observed. However, a significant respiration effect, constituting an increase

[4] The Trit was donated by the Smith, Kline and French Labs, and the Tx by the Glaxo Company of England.

of 70%, is observed in terms of the oxygen consumption per gram of tadpole. It is interesting to note that the metabolic response precedes any significant morphological response. The fall in metabolism noted at the sixth and seventh day may be due to the effect of prolonged non-feeding, since this experiment was extended longer than the other Trit experiments reported herein. Figure 2 shows the effect of a 5-fold increase in dose of Trit and emphasizes the dramatic effects of the hormone on both oxygen consumption and tail length. However, quantitatively, no further increase in oxygen consumption was noted, indicating that a maximum in this response had been reached even with the lower dosage of Trit. These data emphasize the lower threshold of the oxygen consumption response in *R. grylio* tadpoles.

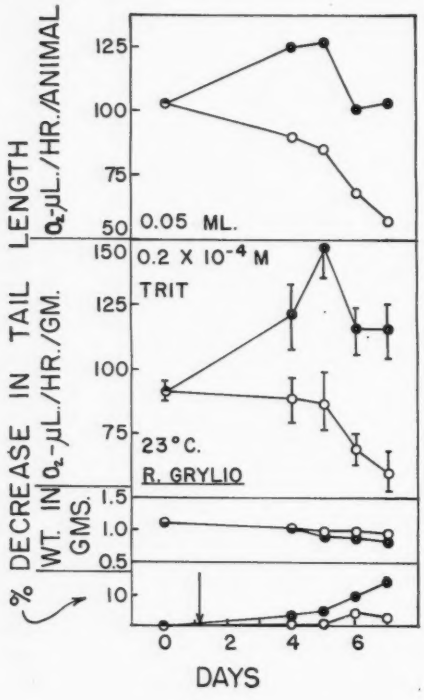

FIG. 1. Effect of Trit on the respiration of *R. grylio* tadpoles at 23° C. The Trit-treated group is indicated by the filled circles. 50 µl. Trit, 2.0×10^{-5} *M*, was injected, as indicated by the arrow, after 27 hrs. from the zero time measurement. The bars in this and the following figures indicate the standard deviation.

The results obtained in the above experiments were compared on the fifth day of each experiment for oxygen consumption per mg. dry weight or per mg. nitrogen (determined at the termination of each experiment, 5–7 days). Calculations of the significance of the differences between the Trit injected group and saline injected controls according to Fishers "t" test gave "P" factors less than 0.1 and frequently less than 0.02. In accord with the results of Etkin (5), there is a tissue dehydration of 40–50% after thyroid hormone treatment. This increase in proportion of dry weight and loss in wet weight was not sufficient to offset the large increase in oxygen consumption. The percentage of nitrogen of the Trit treated tadpoles also increases 25–50%, but, again, insufficiently to account for the increase in oxygen consumption. These data support the view that after Trit treat-

ment there is a significant increase in respiration regardless of what unit of tissue is used to express the results.

It was also observed that other factors such as the age and development of the tadpole, the species and the length of time the animals are held under laboratory conditions influenced appreciably the response of Trit. As far as possible animals with identical histories were used for comparable experiments. In the bullfrog tadpole, *R. catesbeiana*, an injection of Trit (0.10 ml. of 2.0×10^{-4} M) caused only a 35% increase in respiration when compared with the control group on a wet weight basis. The bullfrog tadpoles used in these experiments were probably younger than the *R. grylio* tadpoles and had initial respirations about 50% higher than *R. grylio* animals used in the previous experiments. A steady decline in oxygen consumption per hour per gram was noted during prolonged laboratory storage of the *R. grylio* species used predominantly in the respiration studies. This was accompanied by an apparent increasing sensitivity of the tadpole to moderate doses of Trit.

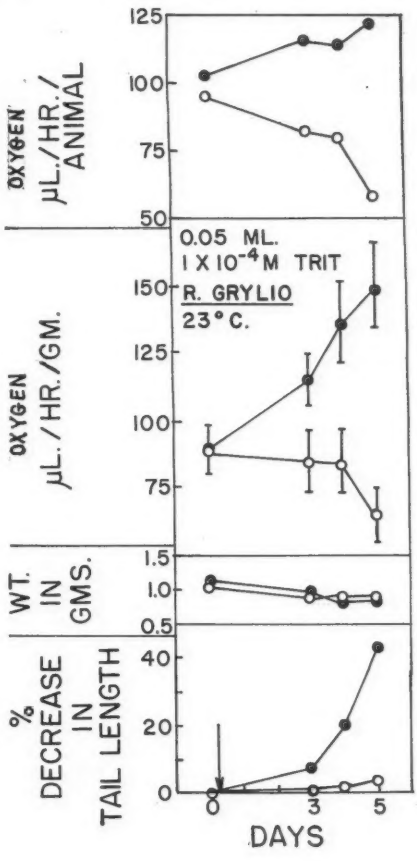

FIG. 2. Effect of a larger dose (50 µl., 1.0×10^{-4} *M*) of Trit on the respiration of *R. grylio* tadpoles at 23° C. The Trit treated group is indicated by the filled circles and a single injection was made as indicated by the arrow, after three hrs. from the zero time measurement.

Five toad tadpoles, *Bufo americanus*, were incubated in 1.0×10^{-7} M Trit at 28° C. A difference in oxygen consumption between the Trit-treated and the controls was noted after 24 hours and reached an apparent maximum in about 48 hours. Respiration increases after 48 hours varied from 40–65% and considerable dehydration was noted.

Effect of Thyroxin.—Larger doses of Tx than of Trit were required for a significant response as shown in Figure 3. It has been noted previously that the response of the tadpole to Trit is probably more rapid and quantitatively greater than to TX (10, 11, 12, 13, 14, 24). As shown in Figure 3, six days were required for a tail decrease of 40%, while less than five days were usually required for an equivalent response to Trit. Regardless, Tx produced a significant respiration increase which, again, appeared to precede the tail response. The decrease in metabolism of the Tx treated tadpoles from the sixth to seventh day is ascribed to the moribund state of the tadpoles, frequently observed at this time when young tadpoles are treated with thyroid hormones. Méhes and Berde (8) noted that Tx caused an increase in the oxygen consumption per gm. of *R. esculenta*, with a maximum on the fourth day after immersion in 1:50,000,000 Tx. The respiration per animal, however, was not consistently increased.

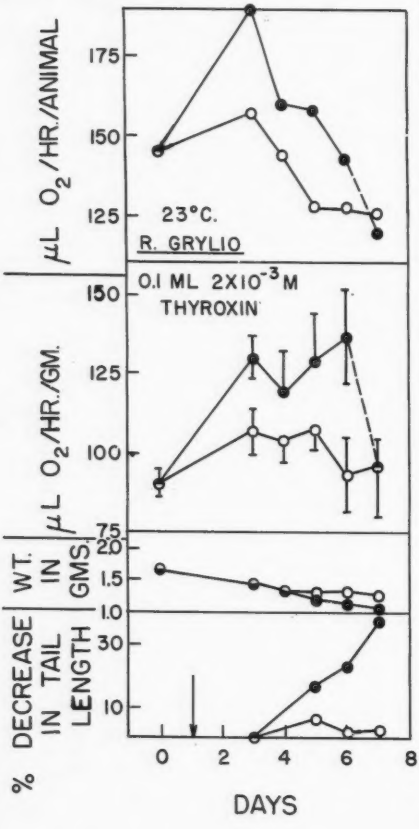

FIG. 3. Effect of Tx (100 μl., 2.0×10^{-3} *M*) on the respiration of *R. grylio* tadpoles at 23° C. The Tx group is indicated by the filled circles and a single injection was given as indicated by the arrow at 24 hrs. after the first measurement.

Respiration During Spontaneous Metamorphosis.—As some of the *R. grylio* animals matured and underwent metamorphosis without the addition of any thyroid hormone, attempts were made to determine their oxygen consumption. However, the process of development is also accompanied by considerable growth and most of these animals weighed between 3–5 gm. It was concluded that respirations determined on such large animals in Warburg vessels of 20 ml. total volume are of questionable validity. In a number of experiments, no correlation between metamorphic stage and μl. oxygen per hour per gram was detected. However, reliable data on the effect of normal metamorphosis on oxygen consumption for

these animals must await the measurement of respiration under more valid conditions.

Data on spontaneous metamorphosis for the smaller toad tadpoles (*Bufo americanus*) are summarized in Table 1. Eight different metamorphic stages have been arbitrarily assigned. The variation in respiration at 23° C per pooled group of five animals is indicated by the standard deviations in the parentheses in several columns. The data indicate no definite trend for

TABLE 1

RESPIRATION OF TOAD TADPOLES DURING SPONTANEOUS METAMORPHOSIS (AT ROOM TEMP.)

STAGE	TOTAL BODY LENGTH	HIND LEG LENGTH	TOTAL NUMBER OF ANIMALS	AVERAGE WEIGHT OF FIVE ANIMALS	NITROGEN	μL. O_2/HR. /FIVE ANIMALS	μL. O_2/HR. /GM.	μL. O_2/HR /MG. OF NITROGEN
	IN MM.	IN MM.		S.D.	IN % S.D.		S.D.	
I	<15	0	45	0.16 (0.01)	0.82 (-)	—	212 (10)	25.8
II	15 - 18	0 - 1	35	0.28 (0.05)	0.81 (0.11)	37	118 (26)	14.6
III	19-21	1 - 2	55	0.52 (0.08)	0.72 (0.40)	73	151.5 (32)	21.0
IV	22-23	2 - 4	35	0.69 (1.00)	0.80 (0.05)	99	145 (37)	18.1
V	>23	>4	60	0.77 (0.10)	0.78 (0.05)	115	151 (31)	19.4
VI	FRONT LIMBS		35	0.68 (0.07)	0.89 (0.20)	109	165 (19)	18.6
VII	SHRINK-ING TAIL		45	0.62 (0.14)	0.91 (-)	113	185 (20)	20.6
VIII	TOADLET		55	0.51 (0.06)	1.06 (-)	123.5	243 (47)	47.6

groups II–V. The increased respiration in group VI and VII may be due to greater activity of these animals since they possess functional limbs. The increase in respiration in the transition from group II to III is regarded as the only possible authentic indication of an increase in respiration during normal metamorphosis. This indicates again that respiration and other metabolic changes precede morphological changes after Trit treatment. This has also been noted in earlier enzyme studies (15, 16).

Effect of Dinitrophenol.—It has been frequently noted that DNP did not emulate the effects of the thyroid hormone in initiating differentiation (17, 18, 8, 15). When *R. grylio* tadpoles were injected with, or immersed in, sublethal doses of DNP, a rapid and significant increase in metabolic rate was observed, as indicated in representative data in Figures 4 and 5. When the tadpoles were injected with DNP and their oxygen consumption

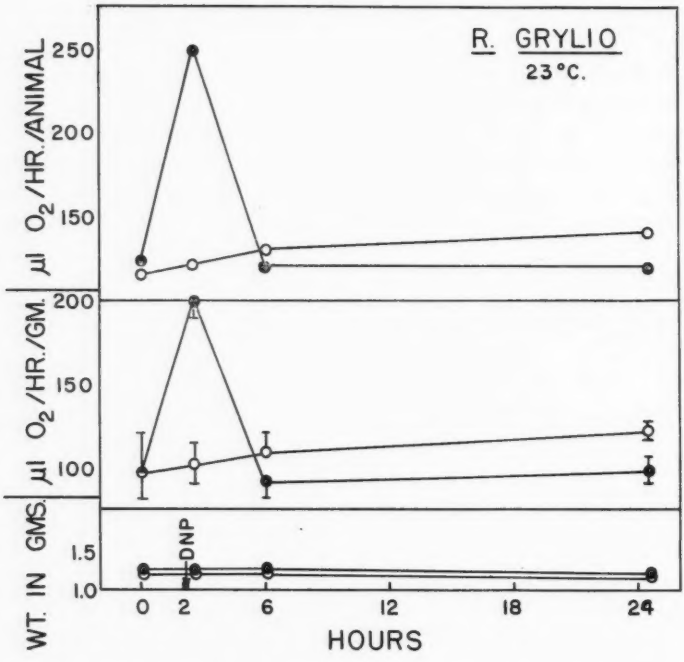

FIG. 4. Effect of injected DNP on the respiration of *R. grylio* tadpoles. The tadpoles were injected with 50 μl., 3.0×10^{-3} *M* DNP (filled circles) two hours after the initial respiration was determined. The respiration was then determined as soon as possible after injection.

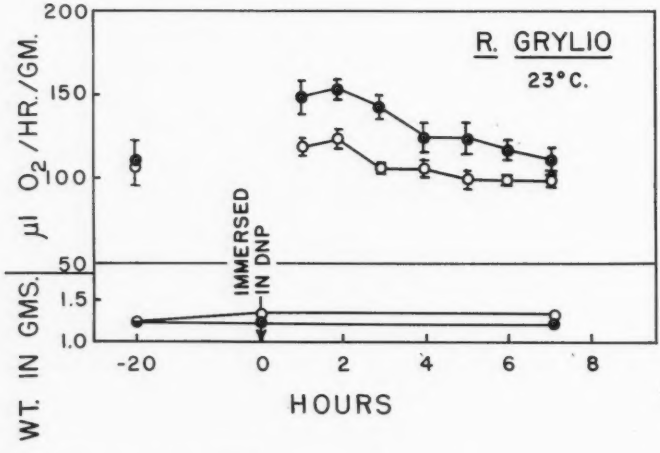

FIG. 5. Effect of immersion in DNP on the respiration of *R. grylio* tadpoles. The DNP group (filled circles) were immersed in 3.0×10^{-5} *M* DNP at zero time.

determined as soon as possible, an increase of over 100% was observed within one hour. By four hours after the injection the average oxygen consumption had fallen to initial values. This experiment furnishes an extremely convenient method for showing the calorigenic effects of drugs. As expected, a smaller but more durable increase in respiration was noted when the animals were immersed in a solution containing the same total amount of DNP. In additional experiments it was found that the response to DNP is a function of the log of the amount of DNP injected. These experiments serve to verify the susceptibility of the tadpole to calorigenic stimuli. The reduction of tail length over a longer period of time during dinitrophenol treatment noted previously (15), may be due to the loss of tail tissues, presumably because of the increased catabolic requirements imposed on the animal.

Méhes and Berde (8) claimed that DNP, 1:400,000 to 1:1,000,000, had no effect on the tadpole's metabolic rate. However a close examination of their data revealed that frequently an increase in oxygen uptake up to 19% was obtained with 1:400,000 DNP. In our experiments, when tadpoles were immersed in DNP (2.2:400,000), a maximum increase in oxygen consumption was observed after three hours. This rapid effect may have been overlooked by Méhes and Berde (8) since they reported measurements only once a day. It is possible that the questionable response after several days exposure to DNP noted by Méhes and Berde (8) was due to the temporary exhaustion of calorigenic substrate in the DNP treated tadpole. While the increase in respiration produced by immersion in DNP are relatively small but significant, there is no question about the much larger and more rapid response to the same amount of injected DNP.

It was also possible to stimulate the oxygen consumption of *R. grylio* tadpoles by the intraperitoneal injection of 1 μg./gm. epinephrine. The increases were erratic and were of somewhat longer duration than those due to an injection of DNP.

Succinoxidase during Induced Metamorphosis.—It has been previously reported that no increases in certain oxidative enzymes have been noted during metamorphosis (6). In experiments on whole tadpole homogenates of the toad and of *R. grylio*, no significant correlation between the stage of normal development and succinoxidase activity has been found (6).

No significant difference was observed in the succinoxidase activity of whole homogenates of normal and Trit treated tadpoles. With no added cytochrome C, eight untreated *R. grylio* tadpoles showed an average succinoxidase activity of 1.7 (standard deviation = ±0.6) μl. oxygen per hour per mg. nitrogen compared with 1.7 (±0.6) for nine Trit treated tadpoles. With 10^{-5} M cytochrome C, the same group of untreated animals gave a value of 3.8 (±0.9) μl. per hour per mg. nitrogen, compared with 4.3 (±1.4) for the Trit group. Much larger differences would be expected between the two groups in order to be statistically significant. Yamamoto and Suzuki (19) have reported a reduction in succinic dehydrogenase

activity in eviscerated *Bufo vulgaris formosus* tadpoles during normal metamorphosis. It is possible that changes in the oxidase part of these systems account for the differences in these data. In any event, in no case was an increase in succinate oxidation or dehydrogenation found in the metamorphosing tadpole.

There are important implications in the absence of a significant effect on succinoxidase following increase in oyxgen consumption after Trit treatment. These effects are invariably associated in rat tissues (7), where the Krebs cycle is believed to be the principal oxidative pathway. Thus there is a possibility that alternative pathways of oxidation, (e.g., hexosephosphate shunt and flavoprotein pathways) not involving succinate oxidation, make a large contribution to the oxidative metabolism of the tadpole. Finally, it should be added that increases in many enzymes have been observed during metamorphosis including liver arginase (15), some proteinases and peptidases (20, 21), and some phosphatases (22, 16).

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EFFECTS OF GROWTH HORMONE AND CORTICOTROPIN ON METABOLISM OF N^{15} FROM GLYCINE, L-ALANINE, AND AMMONIUM CITRATE[1]

O. H. GAEBLER, RACHEL GLOVINSKY, HELEN LEES, DOROTHY KURRIE AND HAROLD C. CHOITZ

Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit, Michigan

ABSTRACT

Metabolism of approximately 20 mg. amounts of N^{15} added to a complete diet as labeled glycine, L-alanine, or ammonium citrate, was studied in normal bitches during nitrogen equilibrium, storage induced by growth hormone, and loss induced by corticotropin.

During induced nitrogen storage, excretion of N^{15} from each of the 3 sources decreased, but the fall was 25, 12, and 9% of the intake respectively for N^{15} from glycine, alanine, and ammonium citrate. Such preferential utilization supports the concept that growth hormone stimulates anabolic processes; it is difficult to explain on the basis of interference with urea formation. An increase in utilization of N^{15} from glycine was particularly noteworthy in the present experiments.

During induced nitrogen loss, output of N^{15} from each of the 3 sources rose. The rise was 10 and 24% of the intake respectively, for N^{15} from glycine and alanine, indicating vigorous catabolism of the latter in experiments with corticotropin.

The partition of excreted N^{15} yielded no evidence that nitrogen catabolism was qualitatively altered by growth hormone or corticotropin. During storage and loss, the expected relationship between urea N^{15} and total N^{15} output was maintained; the minute amount of isotope excreted in creatinine did not change significantly. Growth hormone increased the amount of N^{15} from all sources that was excreted as ammonia, presumably by increasing incorporation of ingested N^{15} into precursors of urinary ammonia.

Incorporation of N^{15} into fibrinogen reached a maximum in 12–24 hours. When the dietary source was glycine, incorporation was large, and essentially the same during nitrogen equilibrium, induced storage, or induced loss. Incorporation of N^{15} from alanine or ammonium citrate increased after growth hormone and decreased after corticotropin, but these changes were small.

ALTHOUGH the effects of growth hormone and corticotropin on the nitrogen balance have been studied extensively, very little is known about the influence of these hormones upon utilization of nitrogen from individual sources. This is somewhat surprising, since balance studies with milligram quantities of N^{15} from individual amino acids have long been

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possible (1, 2). Studies with N^{15}-labeled glycine, involving determinations of the size of the nitrogen pool and rate of protein synthesis (3), and of the rate of amino acid catabolism (4), have previously been carried out in dogs treated with growth hormone (5).

Utilization of N^{15} from glycine is of particular interest, since this amino acid is incorporated directly into many nitrogenous compounds, such as the purines, which are components of nucleic acids. Interest in alanine nitrogen stems from the observation that hepatic glutamic-pyruvic transaminase activity is greatly reduced by growth hormone (6, 7). Whether utilization of ammonia nitrogen is altered by growth hormone or corticotropin is also a question of considerable importance.

In the present study, the total output, rate of excretion, partition in urine, and incorporation into fibrinogen of N^{15} from glycine, alanine, and ammonium citrate were observed in normal dogs, during nitrogen equilibrium, storage induced by growth hormone, and loss induced by corticotropin.

MATERIALS AND METHODS

General Procedure. Two normal adult bitches were used. The quantity of food that each animal required to permit maintenance or slow increase in weight was established during preliminary observations, and fed in a single daily meal. Urine periods were terminated by catheterizing and washing the bladder. Each experiment included a preliminary period of a week or more, during which the daily nitrogen output and weight indicated a state of equilibrium. Occurrence of the anticipated storage or loss was established in each experiment with growth hormone or corticotropin respectively, since determination of N^{15} output involves determination of total nitrogen excretion. A period of at least 2 months elapsed between experiments to permit excretion of ingested N^{15}.

Diets. The basal diet had the following composition, in per cent: casein, 36.2; cracker meal, 36.2; corn oil, 19.6; Phillips Hart (8) salt mixture, 4.0; and yeast, 4.0. Each animal received 2 drops of haliver oil daily. In addition to the yeast of the diet, dog 64 received a mixture containing the following daily allowances of vitamins, in milligrams: thiamine hydrochloride, 0.75; riboflavin, 1.5; nicotinic acid, 7.5; pyridoxine hydrochloride, 0.6; and calcium pantothenate, 3.0.

Preparation and Feeding of Labeled Compounds. Potassium phthalimide containing 32 to 34 atoms per cent of N^{15} was used in synthesizing glycine and DL-alanine (9,10). The latter was resolved (11) to obtain L-alanine. Both labeled amino acids were identified by their nitrogen content and by parallel paper chromatography with authentic reference samples. Labeled ammonium citrate was obtained by distilling an alkaline solution of ammonium nitrate containing N^{15} in the ammonium radical, and collecting the distillate in a slight excess of citric acid. The N^{15}-labeled compounds were fed on the third day of growth hormone, and the second day of corticotropin therapy.

Hormone Preparations and Dosage. Armour growth hormone was used in the glycine experiments on dog 57 (Lot M-10810) and dog 64 (Lot M-208). In all other experiments, we used bovine growth hormone, Lot 50109, List 916, supplied by the Endocrine Study Section, National Institutes of Health. The animals received subcutaneous doses of 5 mg. daily for 4 days. The corticotropin preparation was Armour's Acthar Gel. A total daily dose of 20 units was injected subcutaneously in 10 unit amounts, at 9 A.M. and 3 P.M., for 3 days.

Analytical Methods. Total nitrogen in urine was determined by a micro-Kjeldahl method involving distillation and titration of 3 to 5 mg. of nitrogen. Urea and ammonia were determined according to Van Slyke and Cullen (12). Creatinine was isolated from 23 samples of urine by adsorption on Lloyd's reagent, elution, precipitation as rubidium creatinine picrate, and conversion to creatinine zinc chloride (13). Fibrinogen was determined by the method of Wu and Ling (14). For determination of N^{15} in urine, fibrinogen, creatinine zinc chloride, or amino acids, the procedures of Hiller, Plazin, and Van Slyke (15) and of Sprinson and Rittenberg (16) were employed. Digestion with mercury as catalyst was continued for 18 hours. Preformed ammonia and ammonia from urea were simply concentrated and transferred to Sprinson-Rittenberg tubes. Atoms % N^{15} was calculated from the 29:28 ratio determined in a model 21–401 Consolidated Electrodynamics mass spectrometer. Each sample was scanned. Corrections for air contamination, based on argon, were rarely necessary and always small. The excess of N^{15} was determined by subtracting atoms % found for Matheson prepurified nitrogen.

Accuracy of Values for N^{15} Excess. Although determinations of N^{15} in any given sample are readily reproducible to less than 0.001 atoms %, variations in the value for N^{15} excess in nitrogenous compounds of biological origin present a problem. In 18 experiments described in this paper, N^{15} excess in fibrinogen before ingestion of the labeled compounds varied in a random manner from 0.004 to 0.011 atoms %; the average value was 0.0076, the standard deviation ± 0.0023. Reporting results in atoms % excess magnifies the relative differences between abundances that are near the natural value. Assuming a natural abundance of 0.36 atoms % for N^{15}, the abundance in the above experiments varied from 0.364 to 0.371. However, the fact remains that a value for N^{15} excess as low as 0.008 can vary nearly ± 0.004 in different experiments. Studies on the effect of oxygen (17, 18) on 29:28 ratios led us to consider this source of error. In our determinations, aged hypobromite (16) was used and scans of the nitrogen samples indicated only traces of oxygen. Other factors of this type could produce a small variable error, highly reproducible in any individual sample. Consequently, differences of 0.004 atoms % excess N^{15} are significant only if they occur consistently in a series of determinations.

RESULTS

Total Output and Rate of Excretion of N^{15}. Data obtained in 18 experiments on 2 normal bitches are presented in Table 1. Whether the source of N^{15} was glycine, L-alanine, or ammonium citrate, the total output during 48 hours following ingestion was decreased by growth hormone and increased by corticotropin, but the magnitude of these changes varied with the source of N^{15}. In the 6 experiments with growth hormone, reductions in 48-hour output of N^{15} in the 2 animals, expressed as per cent of ingested N^{15}, were respectively 24.7 and 24.8 for glycine, 10.1 and 14.3 for alanine, and 8.8 and 10.3 for ammonium citrate.

The increase in N^{15} output after corticotropin was much greater when the isotope originated from alanine than when the source was glycine. When N^{15} was derived from ammonium citrate, output during control experiments was very high, and corticotropin did not always increase it. Results for the 2 experiments with ammonium citrate and corticotropin are the only selected data in Table 1; in 2 rejected experiments, excretion of N^{15} was lower, but other responses to corticotropin, such as anticipated

changes in water intake, urine volume, and allantoin excretion, were also small.

Confirming earlier studies (2, 19), we found the increase in fecal N^{15} to be small; during the 48-hour period of observation, it accounted for only about 1% of the amount ingested. Incidentally, fecal N^{15} includes isotope that has been absorbed and re-excreted, for example, in bile pigments (20).

Rate studies (Table 1) in 6 experiments with labeled glycine show that the effect of either hormone upon N^{15} output was very consistent through-

TABLE 1. EFFECT OF GROWTH HORMONE (G.H.) AND CORTICOTROPIN (ACTH) ON UTILIZATION OF N^{15} FROM LABELED GLYCINE, ALANINE, AND AMMONIUM CITRATE, IN NORMAL ANIMALS

Dog No.	Experiment	Gm. N Stored During 2 Days	Labeled Compound	Mg. Excess N-15 Ingested	% of Ingested N-15 Excreted				
					Hours after Ingestion				Total for 2 Days
					0–6	6–12	12–24	24–48	
57	Control	+ 0.50	Glycine	21.65	9.7	17.5	11.3	7.8	46.3
57	G.H.	+ 2.24	Glycine	21.65	3.7	7.8	5.5	4.6	21.6
57	ACTH	− 2.31	Glycine	21.65	10.7	20.7	12.1	8.1	51.6
64	Control	+ 0.61	Glycine	21.65	13.4	15.9	9.2	7.5	46.0
64	G.H.	+ 5.21	Glycine	21.65	6.7	7.1	3.5	3.9	21.2
64	ACTH	− 6.94	Glycine	21.65	15.6	25.9	11.6	6.8	59.9
57	Control	− 0.41	Alanine	19.73	11.9	20.9	11.6	6.2	50.6
57	G.H.	+ 2.82	Alanine	19.73	8.3	14.5	10.1	7.6	40.5
57	ACTH	−10.86	Alanine	19.73	35.2	32.0	11.1	4.1	82.4
64	Control	+ 0.71	Alanine	21.65	15.8	24.8	12.1	12.0	64.7
64	G.H.	+ 4.91	Alanine	21.65	13.3	19.4	10.2	7.5	50.4
64	ACTH	− 8.37	Alanine	21.65	28.8	31.7	13.7	7.6	81.8
57	Control	− 0.35	$NH_4Cit.$	21.46	16.6	36.0	22.3	7.3	82.2
57	G.H.	+ 2.11	$NH_4Cit.$	21.46	30.6	27.6	10.9	4.3	73.4
57	ACTH	− 8.39	$NH_4Cit.$	21.46	46.0	38.1	9.5	2.8	96.4
64	Control	− 0.43	$NH_4Cit.$	21.46	32.5	29.8	14.9	7.2	84.4
64	G.H.	+ 5.34	$NH_4Cit.$	21.46	27.8	30.6	11.9	3.8	74.1
64	ACTH	− 6.80	$NH_4Cit.$	21.46	36.4	39.0	12.6	5.5	93.5

out the period of observation. In experiments with growth hormone, rates of excretion of N^{15} from glycine and alanine were qualitatively similar; when corticotropin was given, excretion of N^{15} from alanine and ammonium citrate was similar, occurring largely during the first 2 periods of 6 hours each.

Large variations in the amounts of nitrogen stored after growth hormone or lost after corticotropin occur, in an individual animal, or in different animals, under identical conditions of treatment. It is apparent (Table 1) that there is close agreement between amounts of isotope excreted by the two dogs, even though the nitrogen stored or lost varies. This suggests that metabolism of a small amount of ingested N^{15} is affected in a more uniform manner than is the nitrogen balance, which reflects changes in metabolism of endogenous as well as exogenous nitrogen.

Nitrogen Partition. Since results for the three N^{15}-labeled compounds were comparable in the 2 animals, and findings for glycine and alanine were similar, data for glycine and ammonium citrate in one animal suffice to indicate characteristic changes in the partition of total nitrogen and total N^{15} between urea and ammonia (Table 2).

In the present experiments with highly purified growth hormone, as in our early ones with crude preparations (21), the fall in nitrogen output was due to a decrease in urea. Consequently, the per cent of total nitrogen ex-

TABLE 2. PARTITION OF TOTAL NITROGEN AND TOTAL N^{15} BETWEEN UREA AND AMMONIA, IN NORMAL DOGS, DURING CONTROL EXPERIMENTS AND DURING TREATMENT WITH GROWTH HORMONE OR CORTICOTROPIN

Experiment	Period	Partition of Total N		Partition of Total N^{15}	
		Urea N	Ammonia N	Urea N^{15}	Ammonia N^{15}
	hrs.	%	%	%	%
Glycine alone	0– 6	84	3.1	85	6.4
	6–12	87	5.2	88	4.5
	12–24	83	5.6	90	3.9
Glycine and growth hormone	0– 6	73	11.0	56	29.2
	6–12	84	11.0	78	14.6
	12–24	64	19.0	59	18.5
Glycine and corticotropin	0– 6	90	4.0	90	4.8
	6–12	89	5.8	94	3.1
	12–24	85	6.6	96	4.1
Ammonium citrate alone	0– 6	(92)	3.5	(102)	1.1
	6–12	87	5.4	96	0.9
	12–24	82	6.4	91	1.3
Ammonium citrate and growth hormone	0– 6	80	5.9	90	2.1
	6–12	82	6.9	92	1.3
	12–24	61	11.4	78	2.6
Ammonium citrate and corticotropin	0– 6	89	3.1	96	0.8
	6–12	84	6.0	92	1.0
	12–24	89	6.6	(102)	1.9

creted as urea fell in experiments with growth hormone (Table 2). During the 12 to 24-hour period, nitrogen partitions as unusual as those which Folin (22) traced to low nitrogen intake resulted from storage of nitrogen, with intake constant. After corticotropin, the percentage of urea nitrogen rose, since the increase in total nitrogen was due primarily to this component.

In control experiments with labeled glycine (Table 2) or alanine, the percentage of total N^{15} eliminated as urea regularly exceeded the percentage of total nitrogen excreted in this form, during the 6 to 12 and 12 to 24-hour periods. Thus the importance of urea as an end product is even more striking than usual, when N^{15}-labeled amino acids are used. As a rule 91 to 97% of excreted N^{15} was recovered as urea plus ammonia. Occasional erro-

TABLE 3. N^{15} EXCRETED AS AMMONIA BY NORMAL DOGS RECEIVING NEARLY IDENTICAL AMOUNTS OF THE ISOTOPE AS GLYCINE, ALANINE, OR AMMONIUM CITRATE

Experiment	Period	Source of isotope, and experimental animal				
		Glycine	Alanine		Ammonium citrate	
		Dog 64	Dog 57	Dog 64	Dog 57	Dog 64
	hrs.	μg.	μg.	μg.	μg.	μg.
Control	0– 6	184	160	123	32	78
	6–12	154	175	193	81	58
	12–24	78	78	76	75	40
Growth hormone	0– 6	385	304	385	135	122
	6–12	166	314	165	125	82
	12–24	126	198	140	80	65
Corticotropin	0– 6	162	205	226	95	65
	6–12	172	165	186	135	85
	12–24	104	90	102	80	50

Values are given in μg. of excess N^{15} per period.

neous recoveries exceeding 100% occur, because the sum is so close to this value, and a product of 2 determinations (gm. of urea nitrogen and atoms per cent of N^{15} therein) enters into the calculation of urea N^{15}.

Changes in the amounts of N^{15} excreted as ammonia were of particular interest. Results for 15 of the 18 experiments shown in Table 1 are presented in Table 3. Growth hormone definitely increased the amount of N^{15} from each of the 3 sources that appeared in urinary ammonia; presumably the hormone increased incorporation of N^{15} into precursors of this excretory product. Effects of corticotropin were neither striking nor consistent.

Although the amounts of N^{15} fed in the experiments presented in Table 3 were nearly the same, excretion of the isotope as ammonia was much greater when it was ingested as glycine or alanine than when it was taken as preformed ammonia. This is due to the well-known fact that ingested

TABLE 4. ATOMS % EXCESS N^{15} IN CREATININE ISOLATED FROM URINE AFTER FEEDING 320 MG. GLYCINE (21.65 MG. EXCESS N^{15})

Hours after Glycine	Dog 57			Dog 64	
	Control	G.H.	ACTH	G.H.	Control
0	.003	.006	.007	.004	.002
3	.005	—			
12	.006	—			
24	.007	.008			
48	.007	.012			
72	—	—	.008	.006	.009
96	.007	.014	—	—	—
168	—	—	.008	.008	—
192	.007	.014	—	—	—
216	—	—	—	—	.010
240	—	.014			
264	—	.014			

ammonium salts are converted to urea almost completely in the liver. That N^{15} from alanine and ammonium citrate behaved differently is especially noteworthy, since isotope from these 2 sources behaved similarly in some of our other experiments.

Since studies on creatinine (Table 4) involved considerable isolation work, investigations on the appearance of N^{15} in this urinary component were carried out only with labeled glycine. Growth hormone profoundly affects utilization of N^{15} from this source, and glycine is incorporated directly into creatine. The concentration of excess N^{15} that appeared in creatinine during control experiments was very small. Less than 0.1% of the total N^{15} excreted during 48 hours appeared in creatinine, while creatinine nitrogen was 2.1% of the total nitrogen. Thus the remote relationship of creatinine to dietary nitrogen, as well as the immediate one of urea, is accentuated when one uses labeled glycine. There was no evidence that either of the hormones altered the minute concentration of N^{15} appearing in urinary creatinine. If one subtracts the "zero hour" value obtained before giving labeled glycine, the highest values in dog 57 are 0.004 atoms per cent excess in the control experiment and 0.008 after growth hormone; in dog 64, in which the order of experiments was reversed, the highest value after growth hormone was 0.004 as against 0.008 in the control. That such small changes have questionable significance has been indicated in the section on Materials and Methods.

Incorporation into Fibrinogen. In each of the 18 experiments presented in Table 1, blood samples for determinations of fibrinogen were drawn before the N^{15}-labeled compound was ingested, and 6, 12, 24, and 48 hours thereafter. The amount of fibrinogen per 100 ml. of plasma and atoms per cent excess N^{15} in fibrinogen nitrogen, were determined in each of the 90 samples, and fibrinogen N^{15} per 100 ml. of plasma was calculated from the 2 determinations.

The average concentrations of fibrinogen in gm. per 100 ml. of plasma, in 3 control experiments, 3 with growth hormone, and 3 with corticotropin, were respectively 0.26, 0.23, and 0.25 in dog 57; and 0.23, 0.22 and 0.20 in dog 64. Thus no definite change in average concentration was produced by the hormones under our experimental conditions. Changes in amounts of fibrinogen N^{15} per 100 ml. of plasma were therefore proportional to changes in atoms per cent excess of the isotope, and contrary to our initial impression (23), yielded no significant additional information.

Atoms per cent excess of N^{15} in fibrinogen reached a maximum in 12 to 24 hours, but the value after 48 hours was still 80–100% of the maximal one. In Table 5 the data have been reduced to simple form by averaging values at 12, 24, and 48 hours, and substracting the "zero hour" value obtained before ingestion of the labeled compound. When glycine was the source, the concentration of N^{15} incorporated into fibrinogen was large, and

TABLE 5. INCORPORATION OF N^{15} INTO FIBRINOGEN IN NORMAL DOGS

Dog No.	Labeled compound	Nature of Experiment		
		Control	G.H.	ACTH
57	Glycine	.033	.030	.032
64	Glycine	.033	.033	.030
57	Alanine	.008	.012	.006
64	Alanine	.009	.013	.005
57	Ammonium citrate	.009	.014	.002
64	Ammonium citrate	.008	.009	.006

Values represent atoms % excess N^{15}.

quite uninfluenced by growth hormone or corticotropin (Table 5) although these hormones markedly alter utilization of N^{15} from this amino acid (Table 1). In the case of N^{15} from alanine or ammonium citrate, evidence that the relatively small incorporation into fibrinogen was increased by growth hormone and diminished by corticotropin is suggestive. In previous experiments with labeled alanine, under quite different experimental conditions, values for atoms % excess N^{15} incorporated into fibrinogen were much larger: 0.044 in untreated, and 0.052 in growth hormone treated hypophysectomized rats (7). Thus an increase in a value that was already high occurred after growth hormone, but extensive N^{15} incorporation took place in the absence of pituitary hormones.

DISCUSSION

The possibility that growth hormone may inhibit one of the enzyme systems involved in urea formation is frequently mentioned. Fraenkel-Conrat, Simpson, and Evans (24) originally found that hepatic arginase activity decreased after large doses of growth hormone; smaller doses, large enough to induce growth, did not lower arginase activity. The present study indicates that the effect of growth hormone on utilization of N^{15} varies markedly when the isotope is derived from different sources such as glycine, alanine, and ammonium citrate. Such preferential utilization would be expected if growth hormone promotes synthetic processes in some way; preferential sparing as a result of interference with urea formation appears less likely.

Incorporation into fibrinogen of N^{15} from uniform amounts of labeled glycine was essentially the same, whether the percentage of ingested isotope retained in the body for 48 hours was 40 to 48, as in corticotropin experiments, or 78, as in those with growth hormone. On the other hand, in several control experiments in which the daily ration was divided, and two doses of labeled glycine, each containing 21.65 mg. excess N^{15}, were fed at 9 A.M. and 3 P.M., the effect was virtually additive; N^{15} excess in fibrin-

ogen rose to 0.056 atoms %. The amount of isotope incorporated into fibrinogen may be determined very early, for example during the first passage through the liver. After the first passage, the liver, muscle, and other tissues compete for circulating amino acids, and the resulting distribution of the isotope would account for the absence of any relationship between percentage of retention and incorporation into fibrinogen.

Catabolism of food nitrogen was obviously increased by corticotropin. In experiments with this hormone, regardless of the compound ingested, most of the increase in excretion of N^{15} occurred during the first two 6-hour periods, during which excretion had also been greatest in the control experiments. It has long been known that catabolism of body protein in fasting adrenalectomized rats is stimulated by adrenal hormones (25). Evidence for an effect on gluconeogenesis has been presented (26), and an enzymatic basis proposed (27). Since catabolism of ingested amino acids also increased, the possibility that corticotropin stimulated gluconeogenesis is clearly not excluded. The behavior of N^{15} from alanine was consistent with this idea, and studies on the fate of N^{15} from non-glucogenic amino acids would be of interest.

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EFFECT OF PROLONGED ADMINISTRATION OF NICOTINE ON THE MEDULLARY VOLUME AND THE DISTRIBUTION OF NORADRENALINE IN THE ADRENALS OF THE RAT, THE MOUSE AND THE GUINEA PIG[1]

OLAVI ERÄNKÖ, VÄINÖ HOPSU AND LIISA RÄISÄNEN

Department of Anatomy, University of Helsinki, Siltavuorenpenger, Helsinki, Finland

ABSTRACT

Rats, mice and guinea pigs were daily injected with sublethal doses of nicotine for 6.5–9 months. Formalin-induced fluorescence and iodate reaction were used to demonstrate the noradrenaline-containing adrenomedullary cells, chromaffin reaction to demonstrate both catechol amines. The medullary volume and the volume of the noradrenaline-containing tissue were determined planimetrically.

In the rat, treatment with nicotine did not affect the adrenal weight but caused an 110% increase of the medullary volume. This increase was mainly due to a 470% increase in the volume of the noradrenaline-containing adrenomedullary parenchyma. No changes in the adrenal weight, the medullary volume or the distributions of the histochemical reactions were detected in the adrenals of the guinea pig and the mouse.

IT HAS been shown that prolonged administration of nicotine causes a hyperplasia of the adrenal medulla of the rat (1) and that the hyperplasia is due to an increase in the volume of the noradrenaline-containing medullary parenchyma (2).

Since these observations were made, a new histochemical method for noradrenaline has been published (3, 4). It seemed to be worth while to reinvestigate the subject using this histochemical reaction, which renders it convenient to estimate quantitatively the volume of the noradrenaline-containing medullary tissue. To study the species differences in the response to nicotine, adrenals of mice and guinea pigs were examined in addition to rats.

MATERIALS AND METHODS

Experimental

The animals were given daily subcutaneous injections of nicotine (Nicotin puriss., Dr. Theodor Schuchardt, G.m.b.H., Görlitz, Germany) in 0.2–0.4% aqueous solution, Sundays excepted.

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Rats were injected for about 9 months, guinea pigs for 8.5 months, and mice for 6.5–9 months. The dose was 0.4 mg. per rat for the first 2 months, then 0.5 mg. per rat. The mean weight of the rats at the onset of the experiment was *c.* 70 gm. and at the end *c.* 200 gm. The mice were given 0.1 mg. per animal and the mean weight of the mice at the end of the experiment was *c.* 30 gm. The guinea pigs were given 1 mg. per animal for the first 2 months, then 2 mg. per animal for 4 months, and 4 mg. per animal for 2.5 months; their mean initial weight was *c.* 240 gm. and the final weight *c.* 720 gm. The surviving animals were killed 2–4 days after the last injection by decapitation.

The iodate reaction

The left adrenals were treated for 24 hours in a saturated solution of potassium iodate for the iodate reaction (4). They were then fixed in 3.5% formaldehyde for another 24 hours and sectioned at 50 μ with a freezing microtome. The total medullary area and the area covered by iodate-positive medullary cells were planimetrically measured from 5 mid-sections of each gland, and an estimate of the relative volume of the noradrenaline-containing medullary tissue was obtained by pooling these values.

The chromaffin reaction

The right adrenals were fixed in a dichromate-formalin mixture and the medullary volume was measured from a complete series of sections, as described earlier (5).

The fluorescence method

Adrenals of other animals were fixed in formol-calcium for the demonstration of the formalin-induced fluorescence (6).

Statistical analysis

To compare the significance of the differences of the means, "Student's" *t*-test was used.

RESULTS

Behaviour of the animals

Marked differences were observed in the immediate responses of the animals to nicotine. The rats regularly showed clonic convulsions *c.* 30 sec. after each injection. These lasted about 1 min., whereafter the animals lay immovable and panted heavily. After a couple of minutes they behaved normally again.

The mice and the guinea pigs did not usually exhibit any convulsions. About 30 sec. after the injection they lay flat and panted, and the guinea pigs salivated heavily. After some minutes, normal behaviour was resumed.

Effect on adrenals

Table 1 lists the adrenal weights, the medullary volumes and the percentages of iodate-positive, *i.e.* noradrenaline-containing, cells in the medulla of the nicotine-injected and the control animals. It will be seen that the rats reacted to the treatment with nicotine by showing a 110% increase in the volume of the medulla, although no significant change in the adrenal weight occurred. By multiplying the medullary volumes by the corresponding percentages of iodate-positive tissue, it was found that the volume of

TABLE 1. EFFECT OF NICOTINE ON THE ADRENALS

Species and treatment	Adrenal weight (mg.)			Medullary volume (cu. mm.)			Iodate-positive tissue (%)		
	No.[1]	Mean	SD	No.[1]	Mean	SD	No.[1]	Mean	SD
Rats									
Nicotine-injected	5	18.7	1.5	5	2.92[2]	0.79	5	31.3[3]	9.7
Controls	5	17.6	3.8	5	1.38	0.40	5	11.8	7.5
Mice									
Nicotine-injected	7	2.2	0.5	12	0.66	0.24	14	16.3	6.6
Controls	5	2.0	0.4	9	0.52	0.10	11	17.6	3.7
Guinea pigs									
Nicotine-injected	7	313	40	7	7.96	1.69	7	0	0
Controls	4	301	63	4	8.02	2.07	4	0	0

[1] The number of glands investigated.
[2] Difference from the mean control value is significant (P~0.005).
[3] Difference from the mean control value is significant (P~0.007).

the noradrenaline-containing medullary tissue in the rat adrenals increased with 470%.

There was also a mean increase in the volume of the iodate-negative medullary tissue, the magnitude of which is of the same order in terms of cu. mm. as the mean increase in the volume of the iodate-positive tissue. However, since the original volume of the iodate-negative tissue is large, the increase is only about 60%. Moreover, this change is not statistically significant and may well be due to a sampling error. Figures 1 and 2 demon-

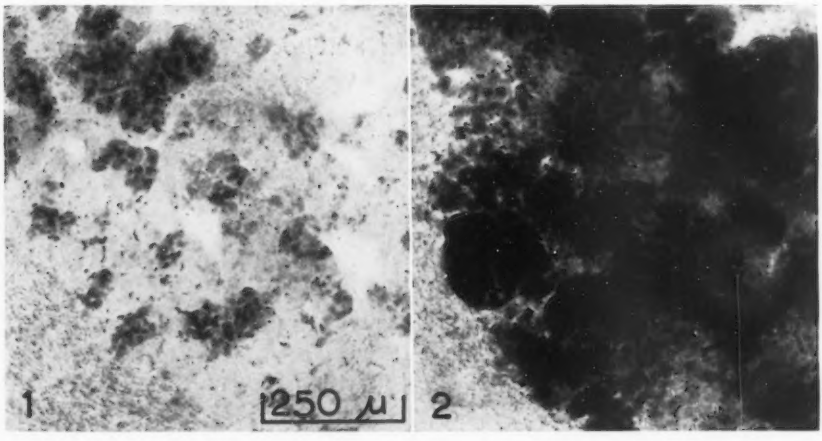

FIG. 1. Adrenal medulla of a control rat. Cortex in lower left corner. Iodate reaction, photographed at 4050 Å in transmitted light. The iodate-positive cell islets in the medulla are clearly visible, the red blood cells black. Magnification *c.* 90.

FIG. 2. Adrenal medulla of a rat injected with nicotine for 9 months. Cortex in lower left corner. A definite increase in the size of the iodate-positive islets can be seen, and the intensity of the reaction is stronger. Technique and magnification as in Fig. 1.

strate that the individual iodate-positive islets increased in size, eventually fusing together, and that the iodate reaction is more intense in the medullary cell islets of the nicotine-injected rats than in those of the controls.

Distribution of the formalin-induced fluorescence was similar to that of the iodate reaction both in the nicotine-treated and in the control rats. However, in contrast to the intensity of this reaction, the fluorescence was weaker in the medullary cells of the nicotine-treated rats than in those of the controls.

The mice and the guinea pigs showed no changes in the adrenal after treatment with nicotine. Differences in the means between the respective experimental and control groups are statistically non-significant and so small that not even a tendency towards changed values in the nicotine groups can be revealed. Also the intensities of the fluorescence and the iodate reaction remained unchanged.

DISCUSSION

The results obtained confirm the earlier observed nicotine-induced hyperplasia in the rat adrenal medulla and show that even under these conditions the two histochemical reactions used for the demonstration of the noradrenaline-containing adrenomedullary cells, *i.e.*, the formalin-induced fluorescence (2, 6) and the iodate reaction (3, 4) run parallel, the fluorescent and iodate-positive tissue being responsible for the hyperplasia. This is what can be expected, since these two reactions have been always found to be distributed in the same way under several experimental conditions (7).

It is of interest that the formalin-induced fluorescence was weaker, the iodate reaction stronger, in the medullary cell islets of the nicotine-treated rats, than in the islets of the controls, although the distributions of these two reactions were identical. Such a discrepancy has been observed earlier (7) and suggests that the two reactions may depend on different properties of the noradrenaline-containing cells, although they both selectively demonstrate them.

Neither the guinea pig nor the mouse adrenals showed any changes. This is somewhat surprising, particularly as far as the mouse is concerned, since the mouse adrenal contains—like the rat adrenal—two types of medullary cells, which the guinea pig adrenal does not, and since the dosage of nicotine given to the mice was higher in terms of body weight than that given to the other two species. In this connection it may be of interest to mention that the nodular hyperplasia induced by nicotine in the rat adrenal medulla is accompanied by pronounced changes in the distributions of cholinesterases and acid phosphatase (8), while no such changes were detected in the adrenals of similarly treated mice or guinea pigs.

Whatever the reason for the difference in the adrenal responses to nico-

tine between the species examined, the present work demonstrates once again that generalizations from one species to another may lead to entirely misleading results.

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THE EFFECT OF TESTOSTERONE PROPIONATE ON THE PROTEIN AND CARBOHYDRATE METABOLISM IN THE DEPANCREATIZED-CASTRATED DOG[1,2]

CHARLES D. KOCHAKIAN AND GIOVANNI COSTA

University of Alabama Medical Center, Birmingham, Alabama and Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma

ABSTRACT

The administration of testosterone propionate at 25 to 100 mg./day for up to 56 days to partially regulated depancreatized-castrated dogs produced a constant increase in body weight accompanied by a retention of nitrogen and a decrease in glucose excretion. The nitrogen effect gradually "wore off" after 3 weeks of androgen injections but the effect on body weight and glucose excretion continued for the duration of treatment. Furthermore, on cessation of injections the nitrogen excretion increased but the glucose excretion gradually returned to the basal level. The blood glucose and NPN changed in parallel with the urinary constituents. The blood amino-N was not affected by androgen administration except for a short period of about 1 week at the end of maximum nitrogen retention and the beginning of the "wearing off" effect on the urinary nitrogen and the blood NPN. The androgen apparently only stimulated the disposition of the surplus glucose. The blood glucose of castrated dogs was not changed by the androgen. The fecal nitrogen decreased on prolonged injection of androgen.

THE protein anabolic action of testosterone propionate can be produced in both the alloxan- and phlorizin-diabetic rat (1). A small but significant nitrogen retention also has been reported after the administration of 25 mg. of testosterone propionate daily for 4 and 6 days to depancreatized bitches maintained on insulin (2). Testosterone propionate, however, was unable to elicit the decrease in blood NPN (3, 4) in the depancreatized bitch deprived of insulin for 24 hours (5). Studies were undertaken, therefore, to obtain further information concerning the metabolic action of the androgen in the diabetic animal. The depancreatized dog was chosen to eliminate the role of the pancreas. Since the rapid deterioration of the depancreatized dog rendered it impossible to carry out extended studies, the diabetes was partially regulated by the administration of insulin. The dogs were castrated because the protein anabolic effect is more readily apparent in the castrated animal (1, 4, 6, 7).

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[1] This investigation was supported by research grant A-914 and A-1726 from the National Institute of Arthritis and Metabolic Diseases, Public Health Service and by the American Cancer Society. Jim Cockrell assisted in these studies.

[2] A preliminary report of some of these data was made before the 38th Annual Endocrine Society Meeting (J. Clin. Endocrinol. 16:933, 1956).

METHOD

Male mongrel dogs were maintained in metabolism cages in a separate air-conditioned room at 70–72° C with artificial light automatically regulated at 12 hours/day. The diet consisted of: horse meat 72.8, raw beef pancreas[3] 12.1, sucrose 12.1, tricalcium phosphate 1.2, Fleischmann's #2019 brewer's yeast 1.2, Wesson's salt mix 0.18 and Patch's cod liver oil 0.36. The constituents were mixed in a food mixer and appropriate amounts for each dog were packaged and frozen. Each batch of diet was analyzed for nitrogen which gave an average value of 2.50 gm./100 gm. diet. Frequent analyses were also made for carbohydrate, moisture and ash. The calculated caloric value of the diet was 210 Calories/100 gm. The frozen food was placed in boiling water and mixed with heating until a uniform mush was obtained. The dogs were fed at 8:00 A.M. and 5:00 P.M. each day and were weighed three times per week. Blood samples were drawn from the femoral vein just before the morning meal.

After a preliminary period of dietary adjustment and training, the dogs were operated.[4] Castration was performed at the time of pancreatectomy. The dogs were again adjusted to a satisfactory dietary level and the insulin requirement determined. The insulin[5] and testosterone propionate[6] were injected twice daily just before feeding.

The urine was collected daily under toluene, analyzed in two day pools and calculated for 7 day periods. Catherization to end the periods was not employed because of the high risk of infection. The feces were collected daily, stored in the refrigerator and weekly pools were homogenized in a large Waring blendor.

Analyses. Nitrogen of the urine was determined by a micro-Kjeldahl procedure. The nitrogen of the food and feces was determined by digestion of quadruplicate samples by the macro-Kjeldahl procedure followed by volumetric dilution and distillation of duplicate aliquots of each sample by the micro-Kjeldahl procedure.

Blood and food reducing sugar was determined by the Somogyi method (8). The food was first hydrolyzed by refluxing with 15% HCl. Urine reducing sugar was determined by the Benedict method (9).

The non-protein nitrogen of blood was determined by both the micro-Kjeldahl and Nessler procedures. The amino nitrogen was determined according to Frame, Russell and Wilhelmi (10).

RESULTS

A total of nine studies with four depancreatized-castrated dogs were run at various dose levels and periods of testosterone propionate administration (Tables 1, 2). The results were essentially the same in every instance but the most uniform and comparable results were obtained when NPH insulin was used to partially control the diabetes (Tables 1, 2; Dogs 2 & 8).[7] The results with Dog 2 under this regimen are presented graphically (Figs. 1, 2).

[3] The raw pancreas was obtained at slaughter and immediately frozen through the generous cooperation of Armour and Company, Oklahoma City.

[4] The operations were performed by Doctors D. Rose and J. Schilling, Department of Surgery, University of Oklahoma Medical School.

[5] The insulin preparations were kindly provided by Eli Lilly and Company.

[6] The testosterone propionate was generously provided by Ciba Pharmaceutical Products, Inc. as perandren, 50 mg./ml.

[7] Blood sugar values were obtained at frequent intervals over 24 hour periods in order to determine the over-all regulation of the carbohydrate metabolism by the various insulin regimens.

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ON THE NITROGEN BALANCE AND BODY WEIGHT OF DEPANCREATIZED-CASTRATED DOGS PARTIALLY REGULATED WITH INSULIN[1]

Dog	Test. Prop.		Body Wgt.		Nitrogen			
						Preinjn.[2]		Injn.[3]
	mg./ day	days	Preinjn. kg.	Max. Gain kg.	Intake gm./day	Urine gm./day	Bal gm./day	Bal gm./day
2	2×25	1–14	14.4	0.0	20.0	10.06	2.36	4.18
	2×50	15–42		2.8				4.88
	2×25	1–21			20.0	13.32	3.97	5.02
	2×50	22–48						3.42
	2×25	1–21	14.3	0.3	20.0	9.86	1.10	2.19
	2×50	22–56		0.7				1.64
	1×25	1– 7	16.1	0.3	15.0[4]	10.24	1.41	0.90
	2×25	8–27		2.1				2.40
	2×50	28–56		3.4				1.50
	2×15	1–28	18.2	0.9	15.0[4]	11.33	0.70	1.69
3	2×25	1–14	15.5	0.0	20.0	10.57	3.28	5.59
	2×50	15–42		1.1				5.95
	2×25	1–21	17.0	1.0	20.0	12.95	1.97	5.87
	2×50	22–48		2.7				6.64
5	2×25	1–21	11.1	0.5	15.0	9.45	0.06	1.57
	2×50	22–56		0.4				0.0
8	1×25	1– 7	14.0	0.0	15.0	12.39	0.58	0.36
	2×25	8–27		2.3				3.87
	2×50	28–56		3.4				0.09

[1] See Table 2 for amount and type of insulin administered and degree of regulation of the diabetes.
[2] Average values for 3–4 periods (weeks) preceding injections of androgen.
[3] Averages of periods showing maximum changes.
[4] Diet reduced 25% and loss of calories compensated by adjustment with 40 gm. primex at each meal.

Studies with two castrated dogs (Table 3) were also made for comparative purposes.

Body Weight. The weight of the dog increased steadily for about 42 days after which it showed a tendency to flatten (Fig. 1). On cessation of injections about one-third of the gain in body weight was gradually lost and a higher basal body weight plateau was maintained. Repetition of the study at a lower dose of androgen and a shorter period of time yielded essentially the same response but with a smaller gain in body weight.

Urine Volume. The water intake was not measured but the urine volume showed no marked variations during the period of injection. On cessation of injections, however, the urine volume increased slightly during the period of weight loss. Hematocrit, RBC and WBC were determined periodically in several of the experiments. No major changes to indicate hemodilution or concentration were observed.

TABLE 2. EFFECT OF TESTOSTERONE PROPIONATE ON THE BLOOD AND URINE GLUCOSE OF THE DEPANCREATIZED-CASTRATED DOG PARTIALLY REGULATED WITH INSULIN[1]

Dog	Insulin[2] I.U.	Test. Prop.		Glucose			
				Blood		Urine	
		mg./d	days	Preinjn.[3] mg./ 100 ml.	Injn.[4] mg./ 100 ml.	Preinjn.[3] gm./ day	Injn[4] gm./ day
2	12N, 12PZI	2×25	1–14	180	107	35.0	33.5
		2×50	15–42		68		8.5
	12N, 12PZI	2×25	1–21	214	145	59.0	59.0
		2×50	22–48		58		40.0
	14N, 14N	2×25	1–21	453	305	62.0	25.0
		2×50	22–56		203		22.0
	7NPH, 7NPH	1×25	1– 7	255	255	32.0	33.0
		2×25	8–27		110		22.3
		2×50	28–56		62		13.3
	7NPH, 7NPH	2×15	1–28	329	150	53.3	33.1
3[5]	12N, 12PZI	2×25	1–14	149	95	34.0	35.0
		2×50	15–42		77		13.5
	12N, 12PZI	2×25	1–21	142	54	20.0	18.0
		2×50	22–56		80		32.4
5	9N, 9N	2×25	1–21	453	447	44.0	29.5
		2×50	22–56		371		29.0
8	5NPH, 5NPH	1×25	1– 7	278	285	20.0	24.0
		2×25	8–27		113[6]		10.0
		2×50	28–56		170		48.0

[1] See Table 1 for additional details.
[2] The figures are A.M. and P.M. dose respectively, N = normal insulin, PZI = protamine zinc insulin, NPH = NPH insulin.
[3] Average values of 2 to 4 basal periods.
[4] Average values of periods showing maximum changes.
[5] Died with convulsions 90 days after the last injection of testosterone propionate and the 380th day after pancreatectomy. Convulsions did not respond to intravenous glucose.
[6] Values during this period were erratic varying between 150–260 mg./100 ml.

Urinary Nitrogen. The dog was in positive nitrogen balance at the beginning of the injections of testosterone propionate due apparently to low basal urinary nitrogen values (Fig. 1). The injection of the androgen, however, produced a further decrease in the urinary nitrogen during the second and third weeks after which the urinary nitrogen gradually increased in spite of the increase in dose of the androgen. On cessation of injections, a further increase in urinary nitrogen excretion occurred followed by a decrease to the true basal level. At this time the experiment was repeated at a lower level of androgen (Fig. 2, Table 1) with a sharp decrease in the excretion of urinary nitrogen after the first week of injections and a sharp increase on cessation of injections.

The urea excretion (Dog 2, expts. 1, 2; Dog 3) changed in parallel with the total urinary nitrogen.

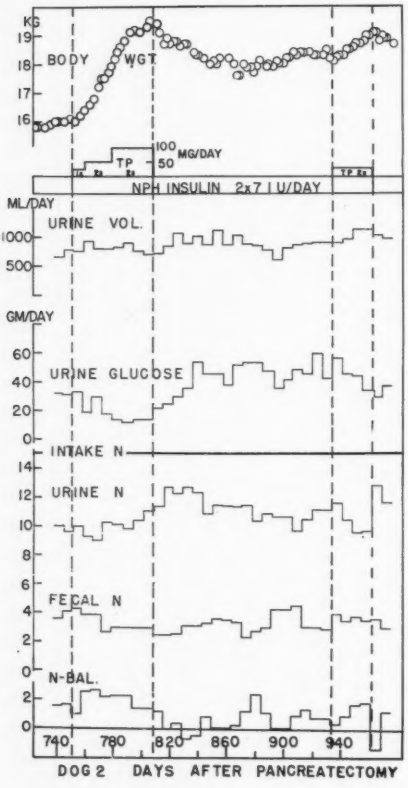

FIG. 1. Effect of testosterone propionate on the urinary constituents and fecal nitrogen of the depancreatized-castrated dog. 2× indicates that the dose of testosterone propionate was divided between the 8:00 A.M. and 5:00 P.M. injections. See Fig. 2 and Tables 1, 2 for other details.

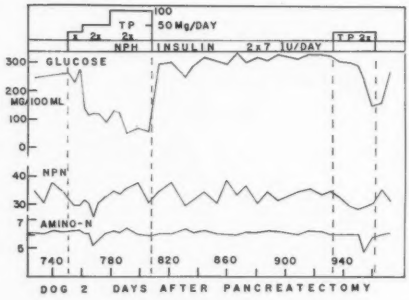

FIG. 2. Effect of testosterone propionate on the basal blood constituents of the depancreatized-castrated dog. The NPN and amino-N are serum values. See Fig. 1 and Tables 1, 2 for other details.

Fecal Nitrogen. The fecal nitrogen excretion was much greater than normal (cf. Table 3). The amount was especially great at the higher levels of food intake (Table 1; Dog 2, Dog 3). Under these conditions the fecal nitrogen was approximately equivalent to the urinary nitrogen and almost identical with those of comparable conditions reported by Chaikoff and

TABLE 3. EFFECT OF TESTOSTERONE PROPIONATE ON THE BODY WEIGHT AND NITROGEN BALANCE OF CASTRATED DOGS

Dog	Test. Prop.		Body wgt.		Nitrogen			
						Preinjn.		Injn.
	mg./d	days	Pre-injn. kg.	Max. change kg.	Intake gm./	Urine gm./ day	Bal. gm./ day	Max. gm./ day
4	1×25	1– 7	12.8	0.0	4.25	3.32	+0.53	+0.87
	2×25	8–15		0.4				+1.18
9	1×25	0–14	15.2	1.0	10.00	8.98	0.0	+1.40
	2×25	15–35		1.0				+2.00

Forker (11). When the diet was reduced and fat substituted to compensate for the loss in calories, the decrease in dietary nitrogen was reflected entirely in the feces (Table 1, Dog 2; Expts. 4 & 5).

After about three weeks of testosterone propionate, the fecal nitrogen gradually decreased to approximately one-half its original value. The reduction coincided with the increase in urinary nitrogen. The decreased fecal nitrogen excretion continued for about three weeks after cessation of injections and then returned to the basal value. This phenomenon was much more striking and prolonged when the dog was receiving the larger nitrogen intake and the fecal nitrogen was greater (Table 1). The fecal nitrogen was gradually reduced to almost one-half and did not begin to return to the original level until about 120 days after cessation of androgen injections. Furthermore, there was a comparable increase in urinary nitrogen and glucose indicating a greater absorption of protein components with a conversion to carbohydrate.

Nitrogen Balance. The changes in urinary and fecal nitrogen are reflected in the nitrogen balance. The androgen induced a further positive nitrogen balance in all of the studies (Table 1, Fig. 1) but on prolonged treatment, the positive nitrogen balance gradually decreased and in some instances was restored to or near equilibrium.

The maximum nitrogen balances produced in the depancreatized-castrated dogs were somewhat greater than those observed in the two castrated dogs (Table 3) and in previous studies with castrated dogs (3, 6, 12, 13).

Urinary Glucose. The injection of testosterone propionate produced a marked decrease in the urinary sugar of all of the depancreatized-castrated dogs (Table 2, Fig. 1) except in experiment 2 of Dog 3.[8] The decrease became apparent one week after the initiation of androgen treatment, reached a minimal level where it was maintained for the duration of androgen treatment (Fig. 1). On cessation of injections, the urinary sugar gradually returned to its basal level (Fig. 1), but at a slower rate than the urinary nitrogen.

Blood Glucose. The androgen produced a response in the post-absorptive blood glucose (Fig. 2) similar to that in the urinary glucose (Table 2, Fig. 1) except that the blood glucose returned more quickly to the original level on cessation of androgen injection (Figs. 1, 2).

The blood glucose level (70–80 mg/100 ml.) of the castrated dogs (Table 3) was not altered by androgen administration.

[8] In the course of this experiment the urinary and also the blood sugar showed wide variations with blood values as low as 25 mg./100 ml. The dog died 90 days after cessation of androgen injections in convulsive seizure which would not respond to intravenous glucose. Autopsy revealed some brain damage which was apparently due to repeated low blood sugar levels produced in the night by the protamine zinc insulin.

Blood NPN and Amino-N. The basal serum NPN in all experiments was gradually decreased on androgen administration but returned towards normal on continued injections (Fig. 2). It is noteworthy that the changes closely parallel those of the urinary nitrogen (cf. Fig. 1).

The amino-N showed a transitory decrease after about two weeks of androgen administration with a rapid return to the original level (Fig. 2).

The basal values for these constituents of the partially regulated depancreatized dogs were essentially the same as those in normal and castrated dogs.

Blood glucose, NPN and amino-N after food and insulin. In order to obtain further insight into the nature of the changes in the blood constituents,

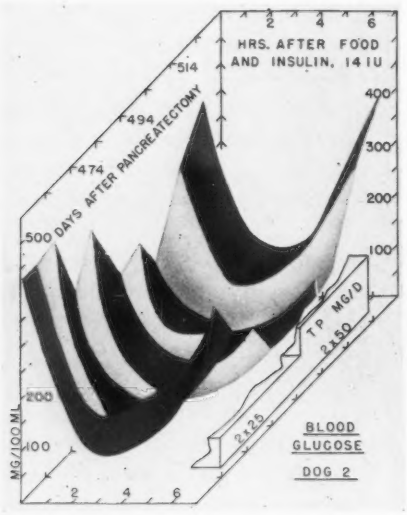

FIG. 3. The response of the blood glucose of the depancreatized-castrated dog to food and insulin during testosterone propionate treatment. See Tables 1 and 2 and Figures 4 and 5 for other details.

these values were determined once per week at hour intervals after the administration of the insulin and the morning feeding in two experiments (Dog 2, expt. 3 and Dog. 5). The dogs ate their food within 15 minutes. The results for Dog 2 are presented in three-dimensional graphs (Fig. 3, 4, 5). The responses in Dog 5 were practically identical to those of Dog 2.

The response of the blood sugar was as to be expected and continued through the period of androgen treatment (Fig. 3). The minimum level to which the blood glucose was decreased, however, was essentially the same in every test in spite of the decrease in the basal values due to androgen administration.

The serum-NPN increased after food and insulin to a maximum level within about 4 hours (Fig. 4). This pattern of response was maintained throughout the period of androgen administration but at the lower level

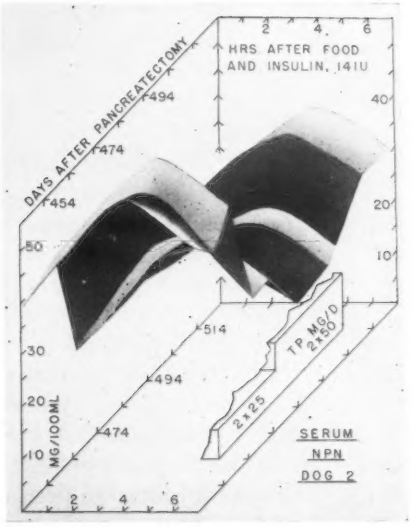

FIG. 4. The response of the serum NPN of the depancreatized-castrated dog to food and insulin during testosterone propionate treatment. See Tables 1 and 2 and Figures 3 and 5 for further details.

induced by the androgen. The absolute maximum increase and time of occurrence remained constant.

The serum amino-N showed a response similar to that of the serum-NPN except that the maximum increase occurred within about 3 hours and the duration of the androgen effect was less.

Other Observations. Ketone bodies in blood and urine indicated no excessive fat catabolism. The average blood values for 3 depancreatized dogs

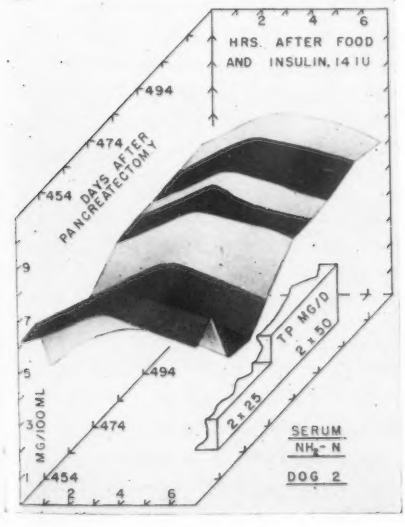

FIG. 5. The response of the serum amino-N of the depancreatized-castrated dog to food and insulin during testosterone propionate treatment. See Tables 1 and 2 and Figures 3 and 4 for further details.

were 1.7–2.2 mg.% and urine 140–190 mg./day which were two to three times those in 2 normal and 1 castrated dog (0.8 mg.% and 45–65 mg./day respectively).

DISCUSSION

These results with the partially regulated depancreatized-castrated dog not only indicate that the pancreas is not essential for the protein anabolic effect of the androgen but also that the androgen has other metabolic properties. Thus, the metabolic status of the animal is an important factor in the revelation of the potential function and action of the hormone.

The body weight increase stimulated by the androgen in the depancreatized dog is more prolonged and thus the final weight change is greater than that stimulated in the castrated dog. This extra increase in body weight cannot be explained by the nitrogen retained. The body weight of the depancreatized dog in contrast to the non-depancreatized dog continued to increase on prolonged injection of testosterone propionate in spite of the "wearing off" effect on nitrogen retention. This latter phenomenon is similar to that in the rat (1, 14) but occurs later in the dog.

An explanation as to the extra increase in body weight may be found in the decrease in blood and urine sugar. The changes in both instances occur in the same manner and are continued throughout the duration of androgen administration. Thus, the androgen supplements insulin's ability to regulate carbohydrate utilization. If it is assumed that the main effect of the androgen on carbohydrate metabolism is to further promote the conversion of carbohydrate to fat (15), then the extra increase in body weight can be attributed to an accumulation of fat as well as protein under the stimulus of the androgen. Indeed, the calculation of retained carbohydrate as fat will account for the difference in body weight between the observed and that calculated from nitrogen retained. Testosterone propionate decreases the ketonemia and ketonuria of both the depancreatized dog (16) and the diabetic patient (17–19). It is of interest that the normal animal without surplus available glucose responds to androgen treatment with an increased utilization of endogenous fat (6, 20 21).

A small portion of the carbohydrate utilized under androgen stimulation may be deposited as glycogen along with the increase in muscle mass (22, 23), and some may be oxidized to compensate for the loss of energy due to the decrease in protein catabolism.

The decrease in urinary glucose after androgen administration can be only partially explained by the decrease in protein catabolism stimulated by the androgen. The urinary glucose and also the blood glucose continue at their decreased levels even while the urinary nitrogen and blood NPN are returning to normal. Furthermore, the urinary and blood glucose levels do not return to basal levels as quickly as the nitrogen constituents. On the

other hand, the alloxan-diabetic rat responds to androgen with parallel qualitative but not quantitative changes in glucose and nitrogen excretion (1) and the thyrotoxic-diabetic patient partially regulated with insulin responds during the 13 days of androgen treatment with a decrease in nitrogen excretion accompanied by an apparently equivalent decrease in glucose excretion (18). Extension of the period of androgen injection may have revealed a difference.

The ability of androgen to stimulate a utilization of carbohydrate apparently is confined only to that above the basal value. The blood glucose level of neither the castrated dog nor of the normal female dog (5) was altered by androgen administration. Furthermore, the ability of insulin to depress the blood sugar level is not supplemented by that of the androgen but rather seems to be replaced. Thus, the level of depression of the blood sugar of the fed-animal by insulin remained the same in spite of the fact that the androgen had greatly decreased the basal blood value. These observations on the role of androgen in carbohydrate utilization are supported by a number of other studies. The ability of androgen to lower the blood glucose level has been reported for the totally depancreatized dog[9] after a single (24) or daily injections (16). Similar results have been obtained in adult (19, 25–27) but not all young (19, 26) diabetic patients. The androgen also lowers the requirement for insulin (24, 29). In the depancreatized dog testosterone propionate at 50–75 mg./day apparently was able to adequately control the diabetes (16).[9] Small doses of the androgen, however, were ineffective in the depancreatized dog (16) and were slightly effective in some but not all diabetic patients on a constant diet without insulin for 2 to 6 weeks (30). It is noteworthy that the hyperglycemia produced by glucagon also is reduced by an androgen (17-ethylnortestosterone) (31).

The blood glucose level of the normal patient as in the case of the normal dog shows no change after androgen treatment (24, 31), Patients with "well-controled diabetes" also seem to respond as normal individuals. The administration of 10, 25 and 50 mg./day of testosterone propionate for 10 days to 13 such patients decreased the sensitivity of one patient and produced insignificant increases or decreases in the sensitivity of the other patients to the Himsworth-glucose-insulin tolerance test (32). A small statistically significant decrease in the arterial but not the venous glucose tolerance curve has been reported in androgen treated normal individuals (33).

The decrease in blood NPN produced by the androgen appears to be a true reflection of the change in urinary nitrogen. Since the major portion of this is urea and urea excretion parallels urinary nitrogen excretion, it would seem that the effect of the androgen is to decrease catabolism of dietary

[9] Unfortunately complete details and data are not presented for these studies.

protein by diverting the absorbed amino acids, peptides, etc., to anabolic processes. The basal amino acid level of the blood on the other hand, is maintained except for a transitory period after about two weeks of androgen administration. It appears that at this point the androgen evokes an extra need for amino acids for some anabolic process. This effect singularly enough occurs at about the time when the maximal nitrogen retaining effect of the androgen from dietary sources has been attained and disappears as the uinary nitrogen retention effect of the androgen "wears off." It should be noted that in contrast to the blood glucose, the absolute increase in the NPN and amino-N of the blood after the ingestion of food is not altered by androgen administration even though the basal values have been decreased.

The decrease in fecal nitrogen with a concomitant increase in urinary nitrogen suggests that the androgen aids in the digestion of the dietary protein possibly by stimulating the secretion of intestinal enzymes. The prolongation of this phenomenon after withdrawal of androgen is somewhat surprising but it should be noted that administration of thyroxine will increase the secretion of intestinal juices which continue at their high rate of secretion for weeks after termination of thyroxine injection (34).

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THE DISTRIBUTION OF ESTROGENS BETWEEN RED CELLS AND SERUM[1]

JOSEPH W. GOLDZIEHER, ROBERT E. BAKER AND MELVIN A. NYMAN

Department of Endocrinology, and the Department of Physiology-Biochemistry, Southwest Foundation for Research and Education, San Antonio, Texas

ABSTRACT

The *in vitro* distribution of C-14 and H-3 labelled estrone, estradiol-17β and estriol between the erythrocytes and serum of horse blood has been studied over concentrations ranging from 0.0001 to 1,000 μg./ml. $30.2 \pm 1.1\%$ of estrone radioactivity, 20.7 ± 2.7 of estradiol-17β radioactivity and $37.3 \pm 3.0\%$ of estriol radioactivity was associated with the erythrocytes at a 50% hematocrit. The distributions were independent of concentration and equilibrium was achieved in less than one hour.

THERE is convincing evidence that the mammalian erythrocyte can bring about metabolic alterations of estrogens (1, 2) and other steroids (3). On these grounds the distribution of steroids in blood, between the erythrocytes and serum, is of considerable interest. In 1932, Kemp and Bjergaard (4) reported that blood estrogens were equally divided between serum and erythrocytes. Albrieux's bioassay studies (5, 6) indicated a 2:1 distribution between cells and serum in nonpregnancy blood and a 1:1 distribution in pregnancy blood. Subsequently Rakoff, Paschkis and Cantarow (7) confirmed the pregnancy blood findings of Albrieux. In a series of papers Bischoff and coworkers (8, 9, 10, 11) examined the solubility of steroids in saline, albumin, and serum as well as the distribution between erythrocytes and serum. These studies were also based on bioassay, with a reported accuracy of $\pm 15\%$ (12) and in most instances the estrogen distribution was merely inferred from changes in the biological activity of the serum phase after equilibration with erythrocytes. In two experiments with rabbit blood, 28 % and 10% of estrone was associated with the erythrocyte phase[2] at a concentration of 35 μg./ml. serum; 17 and 19% of estradiol was associated with the erythrocytes over a concentration range of 0.3 to 80 μg./ml. and 40 and 41% of estriol was associated with the erythrocytes at a concentration of 25 μg./ml. serum. In human pregnancy blood (11) 16% of the total estrone+estradiol and 22% of estriol was associated with the cells. Of interest in these studies was the observation that equilibrium dis-

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[2] All figures refer to equal phase volumes, i.e. a hematocrit of 50%.

tribution was achieved in 10 minutes at 37° C. Sandberg, Slaunwhite and Antoniades (13) added C-14 labeled steroids to human blood and determined the distribution of radioactivity after one hour's incubation. 29% of estrone and 31% of estradiol were associated with the erythrocytes. Intravenous administration of C-14 labeled steroids yielded inconsistent findings: in 3 patients no radioactivity was associated with the erythrocytes after administration of estrone 4-C-14; in 2 other patients erythrocyte-bound radioactivity was observed 15 and 30 minutes after intravenous injection. In another case, however, an initially high erythrocyte radioactivity decreased for 1½ hours after injection but then showed a sustained rise for at least 24 hours. At the same time, the unconjugated (i.e., chloroform-extractable) steroid radioactivity in serum dropped to zero and the conjugated (i.e., glucuronidase-hydrolysable) steroid radioactivity in the serum declined below that of the erythrocytes.

The behavior of other steroids in whole blood appears to be quite different, in spite of the fact that all endogenous steroids so far studied are largely bound to proteins (14, 15, 16). Eik-Nes *et al.* (17) state that 90% of Porter-Silber chromogens are in the plasma but that the steroids penetrate the cells rapidly on standing. Bush (18), quoting the unpublished results of Bibble, states that little corticosteroid is in the cells before ½ hour, but that an equal distribution between cells and serum is attained in 6 hours. These findings are not entirely substantiated by the studies of Sandberg *et al.* (13) who found that 8% of hydrocortisone 4-C-14, 7% of corticosterone 4-C-14 and 9% of desoxycorticosterone 4-C-14 were associated with the erythrocytes after equilibration for one hour *in vitro*. On the other hand, no erythrocyte radioactivity was noted after labeled hydrocortisone had been given intravenously to human subjects.

MATERIALS AND METHODS

Since the present investigation was engendered by studies of estrogen biosynthesis in the perfused horse testis, it was limited to blood from healthy stallions. In the first group of experiments blood was defibrinated in 1 liter batches and stored at 4° C for 24 hours after the addition of 100,000 units of penicillin and 1 mg. streptomycin. Estrone 4-C-14 (2.7 μc./mg.), estradiol-17β 4-C-14 (2.7 μc./mg.) and estriol 4-C-14 (22.2 μc./mg.) were dissolved in ethanol; aliquots representing 0.14 to 0.22 μc. were pipetted into flasks and flash-evaporated at 40° C under nitrogen (conditions for all evaporations). After dissolving the steroid in 0.1 to 0.2 ml. ethanol, 25 ml. Ringer's solution, 100 ml. defibrinated blood and 313 mg. glucose were added to each flask. The preparations were rocked in an incubator at 37° C in a 95% oxygen-5% CO_2 atmosphere for periods up to 8 hours. Samples of 10 or 15 ml. were removed at intervals; a portion thereof was used for the determination of hematocrit in Wintrobe micro-tubes and the remainder was centrifuged at room temperature for 30 minutes. The serum was aspirated and samples taken for determination of hemolysis in duplicate (19). The buffy coat was aspirated from the remaining cells, which were then resuspended in Ringer's solution and centrifuged. After removal of the supernatant solution, the cells were diluted to a known volume with Ringer's solution. Extraction of the estrogens was carried out by a

quantitative technic for total lipide extraction (20). Duplicate 3 ml. aliquots of serum or erythrocyte suspension were extracted four times with 3 volumes of Delsal's reagent (4 parts redistilled methylal to one part methanol). The four extracts were combined in a separatory funnel, and to this material was added an equal volume of diethyl ether, 2.5 volumes of isopropyl ether, and 1 volume of 1% aqueous acetic acid. The mixture was shaken thoroughly and the aqueous phase discarded. The organic phase was washed twice with equal volumes of distilled water and then taken to dryness in the rotating evaporator. The entire residue was dissolved in toluene and the radioactivity of duplicate aliquots measured by liquid scintillation in a Packard Tri-Carb spectrometer. Correction was applied to each sample for quenching and self-absorption by the addition of an internal standard to the sample and recounting. The internal standard consisted of 12,000 cpm of C-14 benzoic acid in 1.0 ml. toluene. All samples were counted to a statistical accuracy of $\pm 5\%$.

In the second group of experiments, involving successive carrier dilutions of estriol, freshly defibrinated blood without added antibiotics was used. Radioestriol dissolved in 0.1 ml. ethanol was diluted to a concentration of 78,000 cpm (3 μg.) per ml. with saline. Carrier estriol was dissolved in a small amount of ethanol and serial dilutions made with saline. For the incubations, 1 ml. of saline radioestriol solution, 1 ml. of the appropriate carrier estriol solution and 8 ml. of fresh blood were combined and incubated under the described conditions for 4 hours. The extraction procedure was identical except that saline was substituted for Ringer's solution. Duplicate extractions of serum, erythrocytes and whole blood were carried out in all instances.

In the third group of experiments, estradiol-17β 6,7-H-3 with a stated specific activity of 3.80 mc./mg. was used. This was diluted without the addition of carrier to a concentration of 0.0026 μg. (12,300 cpm) per ml., and by the addition of carrier and dilution with ethanol to a concentration of 0.26 μg. (153,000 cpm) per ml. Incubations were carried out for a period of 1 hour with the technic described for group 2 above. Randomly-labeled H_3-cholesteryl acetate with a specific activity of 12.6 μc./mg. was used as an internal standard for scintillation counting.

RESULTS

The distribution of radioactivity from the three major estrogens between stallion erythrocytes and serum diluted with Ringer's solution is shown in Table 1. 30.2 ± 1.1 (S.E.)% of estrone radioactivity, $23.3 \pm 2.9\%$ of estradiol-17β radioactivity and $37.4 \pm 3.1\%$ of estriol radioactivity was associated with the erythrocytes. These distributions compare favorably with the earlier bioassay data of Bischoff *et al.* (10) on rabbit blood, but do not resemble the relatively high erythrocyte estradiol of Sandberg *et al.* (13) or the low erythrocyte estriol of Bischoff and Stauffer (11) which were observed in human blood. Examination of the hourly samplings of the various incubations indicates that equilibrium was established within 1 hour and possibly within $\frac{1}{2}$ hour, although the number of samples for the latter time interval are too few to be conclusive. Moreover, with the possible exception of the incubation of estriol with blood sample 2A, there is no consistent tendency for the steroid distribution to increase in favor of the erythrocytes with the passage of time. The differences in the erythrocyte/serum distribution of the individual estrogens are statistically significant at the $P = 0.05$ level or less.

TABLE 1. INCUBATION OF STALLION BLOOD WITH C-14 LABELLED ESTROGENS

Steroid added	Incubation no.	Blood no.	Amount steroid added, μg.	% radioactivity in cells at hour						Mean	Standard error
				½	1	2	3	4	8		
Estrone	1	1A*	0.81	38**	33	27	—	27	36	32	
	2	2A	0.74		32	30	30	37	—	33	
	3	1B	0.74		25	25	—	26	31	27	
	4	2B	0.74		26	35	—	25	—	29	
										30.2	±1.1
Estradiol	5	1A	0.81	17	13	41	—	25	50	29	
	6	1B	0.74		18	21	—	—	26	22	
	7	2B	0.74		18	20	—	15	16	17	
										23.3	±2.9
Estriol	8	2A	0.06		14	40	47	—	—	34	
	9	2A	0.06		37	43	—	57	—	46	
	10	1B	0.06		49	39	—	35	40	41	
	11	2B	0.06		27	—	—	28	30	28	
										37.4	±3.1

* Numeral identifies the experimental animal; letter identifies particular blood sample from that animal.
** Calculated for a 50% hematocrit.

In the experiments summarized in Table 1 substantial quantities of estrone and estradiol-17β (0.74 to 0.81 μg./ml.) were necessary to provide sufficient counts for statistical accuracy. Although these concentrations approach the physiological range far more closely than the levels generally used in experiments based on bioassay, they were still not satisfactory from this point of view. Since experiments at high dilutions and also at high concentrations might yield results of theoretical significance, further studies were undertaken as described in the second and third group of experiments. Table 2 summarizes the erythrocyte/serum distribution of estriol radioactivity over a concentration range from 0.3 μg./ml. to 1,000 μg./ml. of horse blood. Within the limits of accuracy of our technic, no significant difference was observed over a three thousandfold range. The high solubility of estriol in blood is not surprising in view of the high dispersing power of serum albumin for estrogens. Sandberg *et al.* report a

TABLE 2. INCUBATION OF STALLION BLOOD WITH VARIOUS CONCENTRATIONS OF C-14 ESTRIOL

Incubation no.	Estriol concentration, μg. ml.	Radioactivity in erythrocytes, %*
12	0.3	49
13	2.3	46
14	5.3	47
15	10.3	48
16	50.3	51
17	100	45
18	500	46
19	1000	51

* Calculated for 50% hematocrit.

dispersing power of 6.0 μg./ml. for 1% human serum albumin at 5° C for estrone, and 30.0 μg./ml. for estradiol under the same conditions. Estriol has not been similarly investigated, but may be expected to have a still higher solubility and dispersibility.

Table 3 summarizes the studies of erythrocyte/serum distribution of radioactivity at high dilutions of tritium estradiol-17β. At the highest dilutions such as 0.0001 μg./ml. blood, the added estrogen probably does not contribute significantly to the level of endogenous estrogen already present. Although the variation is considerably greater in this group of experiments

TABLE 3. INCUBATION OF STALLION BLOOD WITH VARIOUS CONCENTRATIONS OF 6,7-H-3 ESTRADIOL-17β

Incubation no.	Estradiol-17β concentration, μg./ml.	Radioactivity in erythrocytes, %* ±S.E.
20	0.0001	8.5±5.7
21	0.01	10.8±3.4
22	0.1	18.9±1.8
23	1.0	16.6±2.6
24	10.0	15.5±3.2
25	100.0	12.3±4.1

* Calculated for a 50% hematocrit.

than in group 2, the differences do not reach a level of statistical significance. If the mean value for erythrocyte radioactivity at concentrations from 0.1 to 100.0 μg./ml. (15.9±3.7) is compared to the mean value for the two lowest concentrations (9.6±4.5) by the most stringent statistical tests, P is found to be greater than 0.2. Thus in this experiment it has not been possible to demonstrate significant changes in the erythrocyte/serum distribution of estradiol-17β radioactivity over a range of concentrations from 0.0001 to 100 μg./ml. horse blood.

DISCUSSION

It must be emphasized at the outset that all the distributions reported in Tables 1–3 refer to the radioactivity of the samples, and that no rigorous proof of the identity of the radioactivity with the actual estrogen in the sample has been attempted. In numerous experiments conducted during evaluation of the extraction procedure, it was shown that the radioactivity of blood samples was quantitatively extracted by this solvent system; hence it is safe to say that the results mirror the distribution of added estrogen together with any metabolites which may have been produced during the period of incubation. Estronase, the only steroid-metabolic system known to be present in erythrocytes, appears to be relatively feeble. Rabbit cells convert estrone to estradiol only to the extent of a few % in 2 hours (21); unpublished studies by one of us (M.A.N.) indicate that fresh human erythrocytes incubated with estrone require 18 hours to convert

50% to estradiol. Thus it is unlikely that our distribution studies of estrone are greatly altered by the estronase reaction, even though studies of estronase activity in the horse have not been carried out.

The partition of steroids between erythrocytes and plasma is said to depend entirely on classical solubility considerations (8, 22, 23). Since albumin is quantitatively the major protein constituent of serum, it may be expected to exert a major influence on the distribution. According to Sandberg *et al.* the dispersing power of 1% human serum albumin is 6.0 μg./ml. for estrone and 27.3 μg./ml. for estradiol. On this basis, one would expect to find a higher proportion of estradiol than estrone in the serum phase, assuming constant affinity in the erythrocyte protein. Our data do, in fact, fulfill this expectation in agreement with the observations of Bischoff but in contrast with the findings of Sandberg *et al.*, who observed approximately equal distributions of estrone and estradiol. The increased proportion of estriol found to be associated with the erythrocytes is difficult to explain in the absence of adequate data on the solubility and dispersibility of this compound in electrolyte and albumin solutions respectively. If polarity is in large measure responsible for the high solubility of corticosteroids in aqueous solution and the relatively low concentration in erythrocytes, one would expect by analogy a similar shift for estriol as compared to the other estrogens, whereas the reverse is actually observed.

The constancy of estriol distribution even at high concentrations is worthy of note. Theoretically one might expect the strong binding sites of the protein to become saturated, and weaker sites with a lower association constant to begin to bind steroid at some point. The constant distribution would seem to indicate that this phenomenon does not take place to a significant degree under our experimental conditions, or that it takes place proportionately in the erythrocyte proteins as well as the serum proteins. At all events, the substantial fraction of each estrogen associated with the erythrocytes at any concentration emphasizes the cells' importance as a transport mechanism for these particular steroids in their unconjugated form.

With the extreme dilutions of tritium estradiol-17β made possible by its high specific activity, it seems reasonable to assume that the circulating concentration of estradiol-17β was not greatly altered. All the available evidence indicates that this added material equilibrates rapidly with the endogenous steroids. The absence of any significant rise in the proportion of estradiol-17β associated with the erythrocytes at very high dilutions suggests that selective adsorption to the erythrocyte cell wall (as another solvent factor in addition to solubility and protein binding) does not occur, or at least does not play an important part at the concentrations studied. However, one cannot on this basis assume an absence of any interaction between the steroids and the erythrocyte. As a matter of fact, Kunz (24)

has shown that a number of steroids other than cholesterol can influence the resistance of the erythrocyte against hemolysis by hypotonic solutions. Nonpolar steroids were much more effective in this regard, while estradiol showed the least potency.

The alteration of the erythrocyte/serum distribution of corticosteroids upon standing, as noted by Eik-Nes *et al.* (17) and by Bibble (cf. 18), cannot be explained by any of the above considerations. The gradual permeation of the cells by corticosteroid suggests either an alteration of cell permeability or a change in the binding power of the proteins governing the steroid distribution. Since no evidence has been adduced for the existence of a permeability factor either by our studies or those of others, the latter possibility appears to be more promising. One is inclined to wonder whether the state of oxygenation of the erythrocyte, through various possible mechanisms, does not affect the intracellular concentration of corticosteroid.

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THE THERMOGENIC INFLUENCE OF PROGESTERONE IN OVARIECTOMIZED COWS

T. RANDALL WRENN, JOEL BITMAN AND JOSEPH F. SYKES

Dairy Cattle Research Branch, Nutrition and Physiology Section
Agricultural Research Center, Beltsville, Maryland

ABSTRACT

A technique for the continuous body temperature measurement of diary cows was devised using a thermistor probe and strip chart recorder. The thermogenic response elicited by progesterone administration to spayed cows (11 trials) was found to be quite marked. More immediate temperature elevations occurred when progesterone was administered intraperitoneally than when given subcutaneously. The advantage of continuous temperature recording in comparison to single daily observations was demonstrated.

RECENT investigations (1) in this laboratory of body temperature variations in normal dairy cows during the estrous cycle have suggested that progesterone elaboration by the corpus luteum may be the factor responsible for elevations of body temperature. In these investigations body temperature was observed to follow the expected development and retrogression of the corpus luteum, being lowest just before estrus, low at the time of ovulation and high during the luteal phase of the cycle.

Similar fluctuations observed in women throughout the menstrual cycle have been ascribed to variations in the titer of progesterone and/or progesterone-estrogen ratios (2–5). Exogenous progesterone has been found to cause an increase in the body temperature in intact and ovariectomized women (3, 6, 7), and in ovariectomized rats (8, 9). Indirect evidence of ovarian hormonal influence on body temperature also exists. Monophasic temperature patterns are seen in postmenopausal (5, 7, 10) and castrate (3, 10) women, pre-puberal girls (10), and in men (11). Frequently, also, disturbances in the temperature curve are seen in women with ovarian insufficiencies (2, 3, 6) as diagnosed by vaginal smear, endometrial biopsy, and amenorrhea. These observations imply that a functioning ovary is necessary for the existence of biphasic curves. Further information in support of the theory that endogenous progesterone is responsible for the postovulatory temperature rise in women is provided by the relationship of this shift to urinary pregnanediol excretion values (3, 4). A close correlation has been found to exist between systemic plasma progesterone levels and waking temperatures during the menstrual cycle (12).

Although there is considerable agreement among investigators that

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progesterone is responsible for cyclic body temperature variations in women, few observations have been made in other species. Our previous investigation (1) indicated that progesterone caused a rise in body temperature in cows; but since these observations were determined by single daily temperature measurements, no quantitative measure of the effectiveness of progesterone could be made. The purpose of the present study was to measure quantitatively the extent of the thermal response caused by progesterone.

EXPERIMENTAL PROCEDURE

Continuous vaginal temperature recordings were made in trials on four ovariectomized dairy cows. In each of 11 trials, after a preliminary control period of several days, the cows were injected with progesterone in an olive oil solution. In seven instances progesterone was administered as single subcutaneous injections of 250 mg., and twice similar amounts were given by the intraperitoneal route. In two other trials the progesterone was given in consecutive daily subcutaneous doses of 150 mg. for two and three days. The minimum time between successive trials in individual cows was sixteen days.

The temperature measurement was accomplished by using a resistance thermometer[1] of the thermistor bridge type and a 0–1 millivolt strip chart recorder[2] connected to the bridge by means of an impedance matching arrangement. The resistance values used were determined experimentally and the precision potentiometer permitted adjustments to give 9° F over the span of the recorder. The accuracy of the bridge as listed by the manufacturer was $\pm 0.18°$ F, and laboratory observations with the thermistor probe in a constant temperature confirmed this.

An 8-inch length of plastic insemination tube was attached to the end of a thermistor probe of rectal-esophageal type by means of 3 one-half inch pieces of small bore rubber tubing to provide stiffening to the probe. This splinted probe was inserted vaginally to a depth of eight and one-half inches and maintained in this position by attaching strings from the protruding end of the plastic rod to the skin of the cow. The strings were tied to 3-inch squares of cheesecloth, and these glued[3] to closely clipped skin areas just medial to the pinbones. Thermistor probes thus applied stayed in position for periods of a week or more without further attention.

Since normal diurnal variations in body temperatures tend somewhat to obscure the significance of sustained elevations in body temperature, our data has been computed as a "thermal index." This is an expression of both intensity and duration of temperature deviations from the daily mean temperature for a two to four day pre-treatment period.

The thermal index was computed in the following manner. Hourly temperatures were read from the chart of the recorder and transcribed on graph paper of five graduations per inch, each vertical graduation being equivalent to one hour and each horizontal graduation being two-tenths degree Fahrenheit. On the graph of each trial a line was drawn indicating the mean temperature for the pre-injection period. The areas of both the positive and negative deviations from this base line were then measured using a planimeter.[4] The algebraic sum of these areas was recorded as the thermal index, and

[1] Model 45 Tele-Thermometer, Yellow Springs Instrument Company, Yellow Springs, Ohio.

[2] Weston Recorder, Model 6701, Type 1, Weston Electrical Instrument Corporation, Newark, New Jersey.

[3] Pliobond Goodyear Adhesive, W. J. Ruscoe Company, Akron 1, Ohio.

[4] Ott Compensating Planimeter, Type 16, Eugene Dietzgen Company.

expressed directly as vernier units of the planimeter readings. A thermal index value was determined for each pretreatment day as well as for post-treatment days. Days were not chronological but were timed from the hour of injection of progesterone. This method of expression of temperature effects is similar to that which has been used in studies of the pathogenesis of fever and tolerance to bacterial pyrogens (13, 14).

RESULTS AND DISCUSSION

A typical record of the continuous measurement of temperature of an individual cow (trial No. 8, Table 1) during control and treatment periods is shown in Figure 1. The base line for all 7 days is the pretreatment mean temperature, 100.80° F. The form of the shadowgraph results from plotting the hourly course of the temperature as it deviates both above and below this control base line. The areas so defined are then expressed in daily thermal index units as described above.

The deviations shown in Figure 1 during the three-day control period are about equal in their positive and negative declinations. After the injection of progesterone, about midway through the first day, the temperature rose to heights significantly above any recorded previously during the control period. Day 2 postinjection shows the maximal effect of progesterone; while day 3, although still showing temperatures consistently above the pretreatment mean, gives evidence of less effect. On day 4 the shadowgraph again resembles the control pattern.

These sustained elevations of body temperature represent a significant effect of progesterone which is not readily apparent from single temperature determinations. For example, in Figure 1 temperature measurement at 4 P.M. would show only a slight rise on the 2nd and 3rd days after progesterone treatment. Measurement of the temperature during the low portion of the diurnal pattern appears to be somewhat better as an index of progesterone action. Thus, measurement at 11 P.M. would show a rise of 0.5–0.6 degree for the first 3 days of progesterone treatment.

The response of spayed cows to injections of progesterone is shown in Table 1. Comparison of the thermal index values for pre-injection days with those for post-treatment days indicates that a thermogenic effect was

TABLE 1. RESPONSE OF SPAYED COWS TO INJECTION OF PROGESTERONE

Trial no.	Cow no.	Thermal index units for control days				Progesterone injections, number, amount, and route	Thermal index units for post-treatment days			
		4	3	2	1		1	2	3	4
1	N291			−20	+ 17	3×150 mg. s.c.	+157	+241	+402	+421
2	N291				− 15	1×250 mg. s.c.	+133	+180		
3	N291	+100	−40	−42	+ 25	1×250 mg. s.c.	+108	+192	+ 67	+171
4	N291	− 57	+34	+20	+ 10	1×250 mg. s.c.	+ 67	+ 60		
5	N291		+ 9	+ 1	− 29	1×250 mg. s.c.	+ 89	+188	+ 69	+ 18
6	N293	− 12	−88	−23	− 21	2×150 mg. s.c.	+262	—	+216	
7	N293	− 71	+45	0	+ 16	1×250 mg. s.c.	+ 89	+ 70	− 2	
8	N293		−52	+46	+ 21	1×250 mg. s.c.	+ 92	+210	+172	+ 58
9	N293		−22	−10	+ 24	1×250 mg. i.p.	+153	+ 74	+ 79	+ 45
10	N1057			+12	− 21	1×250 mg. i.p.	+149	+ 95	+ 63	
11	2643	+ 96	+17	+21	−132	1×250 mg. s.c.	+260	+308	+323	+115
Mean		+ 11.2	−12.1	+ 0.5	− 9.5		+141.7	+161.8	+154.3	+138.0

elicited in every instance. The thermal index during the control days varied about the mean, the largest observed deviations being 100 in the positive direction and 132 negatively. Following treatment with progesterone all of the deviations were positive, with the maximal thermal indices most often occurring on day 2 postinjection in those instances in which subcutaneous doses were administered (trials 1, 2, 3, 4, 5, 6, 7, 8, and 11). Examination of the diurnal patterns of these individual trials indicates that a latent period of 12–18 hours exists between injection and the temperature increase. Thus the rise which occurred in these trials started in the latter part

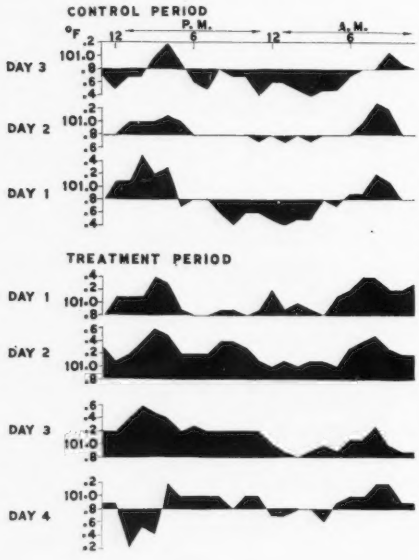

FIG. 1. A typical record of the continuous measurement of temperature of a cow, showing deviations in temperature from the control period mean both before and after treatment with progesterone.

of day one after progesterone administration. In two instances, trials 1 and 6, injections were repeated on successive days. The increases effected were somewhat larger than with single injections.

The existence of a time interval of 12 or more hours following the subcutaneous hormone injection before the temperature effect became noticeable suggested the possibility that progesterone was being slowly absorbed. It is known that the route of administration of pharmacological substances greatly influences the rate of absorption and consequently largely determines the latent period between administration and onset of action, and the intensity of action (15).

In experiments reported by Selye (16), in which progesterone and other steroids were administered to rats, marked differences in toxicity were noted with different routes of administration. Thus subcutaneous injections of progesterone were well tolerated with no ill effects, whereas equiv-

alent injections given intraperitoneally caused anesthesia or death. Similar results with rats have been obtained in this laboratory. Selye theorized that the effects were produced by the more rapid absorption via the intraperitoneal route. In view of these experiments it seemed desirable to administer progesterone intraperitoneally in an attempt to determine if the latent period of thermogenic action in spayed cows could be altered.

Intraperitoneal injections (trials 9 and 10, Table 1) resulted in considerable shortening of the latent period, and the thermal index values reflect this faster effect. In these trials the maximal effect occurred on day one following treatment, whereas in most of the trials in which subcutaneous injection was employed the maximal response was elicited on the second day. The temperature increasing effect was noticeable two to four hours after the injection of progesterone. This short latent period is in marked contrast to the 12–18 hour latent period observed on subcutaneous administration.

The bovine is similar in its thermogenic progesterone response to that of other species studied (3, 6–9). In all probability progesterone elaborated by the corpus luteum is responsible for the pattern of body temperature fluctuations which occur with the estrous cycle in normal cows (1, 10).

Acknowledgments

The authors wish to express appreciation to the staff of the Farm Electrification Branch, A.E.R.D., Beltsville, Maryland, for their assistance with the instrumentation techniques utilized.

We are also grateful to A. J. Kral, Jr., of this laboratory, for his technical assistance.

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DIFFERENTIAL RESIN BINDING OF INSULIN IN SERUM[1]

MARVIN L. MITCHELL, WILLIAM O. WHITEHEAD[2] AND MARY E. O'ROURKE

Department of Medicine and Radioisotope Unit, Lemuel Shattuck Hospital and Tufts University School of Medicine, Boston, Massachusetts

ABSTRACT

A method of interaction analysis by the differential resin adsorption of insulin has been designed which quantitated the binding of insulin by sera *in vitro*. Sera containing insulin-I^{131} were equilibrated in tubes with measured volumes of anionic resin. Following equilibration the tubes were removed and the radioactivity was measured in a well-type scintillation detector. The resins were then washed with distilled water in order to remove the radioactivity that was not resin bound and the tubes were recounted. The radioactivity remaining on the resin divided by the initial radioactivity measured the fraction of hormone bound by the resin ("resin index"). A low resin index reflected increased binding of hormone by serum, and vice versa. Increased binding of insulin-I^{131} by the sera from insulin responsive diabetics was demonstrated when compared with sera from normal subjects. The ability of the resin to bind insulin in serum depended to some extent upon factors other than the competitive binding of insulin by the serum proteins. There was no relationship between the degree of insulin binding by sera from insulin treated diabetics and insulin tolerance.

DEVIATIONS from the normal in the behavior of I^{131} labeled insulin in serum from insulin treated subjects have been demonstrated both *in vitro* and *in vivo* (1, 2). The alterations, which result in a prolonged retention of circulating insulin-I^{131}, as well as its association with specific plasma proteins, have been attributed to insulin binding antibodies (1). Furthermore, it has been postulated that such antibody binding might hinder the tissue utilization of insulin sufficiently to produce an apparent state of insulin insensitivity (2). Whether insulin binding as such has any role with regard to insulin requirement in insulin responsive diabetics has been the subject of considerable conjecture (1–4).

In order to define further the relationship between serum-insulin binding and insulin need, an *in vitro* technique has been developed which permits quantitation of the differential resin adsorption of labeled insulin in serum. In this procedure the assumption is made that the insulin that is not already bound to serum protein is taken up by the resin binding sites which have not been saturated by other serum components. This paper will de-

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[2] Present address: Tufts University School of Medicine, Boston, Massachusetts.

scribe the methods and the results of measurements of insulin-binding on sera obtained from several different groups of subjects.

METHODS AND RESULTS

Preparation of the resin

Amberlite IRA-400,[3] a strongly basic anion exchange resin, was washed by decantation with several volumes of glass double distilled water until the supernatant was free from discoloration and particulate matter. One molar ammonium acetate was added in a volume ratio of eight to one of the wet resin. The mixture was stirred for two hours at 3° C. Following this, the buffer was replaced by fresh molar ammonium acetate and the resin was allowed to equilibrate in the cold until the pH of the slurry remained constant. As a rule, the latter step was carried out overnight, and the final pH of the resin was 7.0 to 7.2. The resin was washed again with distilled water until the washings were clear and colorless. Before its use, the resin was poured as a slurry into a sintered glass funnel (medium) and the excess water was permitted to drain by gravity. Some of the wet resin was transferred to a glass plate where a portion of it was introduced part-way into a 10 ml. syringe with the aid of the flat side of a spatula blade (Diagram 1).

The end of the syringe barrel had been removed at the 1 ml. graduation in order to facilitate the insertion of the resin. Uniform packing was achieved and the excess water expressed by placing the open end of the syringe against the glass plate and pressing the plunger down against the head of the resin. One ml. volumes of the damp resin were then measured into graduated pyrex test tubes (16 mm.×100 mm.) by cutting the resin with the spatula blade as it was extruded from the syringe (the first and last ml. in each syringeful of resin were discarded). The samples were washed repeatedly with distilled water until the supernatants (removed by aspiration) were no longer turbid.

Determination of resin index

Duplicate 1 ml. aliquots of serum were equilibrated for a minimum of 15 minutes with approximately 0.02 microcurie of I^{131} labeled insulin and less than 150 microunits of stable insulin[4] and then were added to the resin tubes. The tubes, inclined at a 30 degree angle to the horizontal, were shaken at 60 oscillations per minute, for one and one-half hours at 3° C.

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[3] The resin was shipped in the chloride form. Cycling the resin between 2.5 N sodium hydroxide and 2.5 N acetic acid before the buffering with ammonium acetate made no difference in the results.

[4] Abbott Laboratories, Oak Ridge, Tenn., specific activity of the insulin when shipped, 5–8 mc. per mg. Upon receipt, 1 to 3 ml. of 1% human serum albumin was added to the container which was then stored at 3° C.

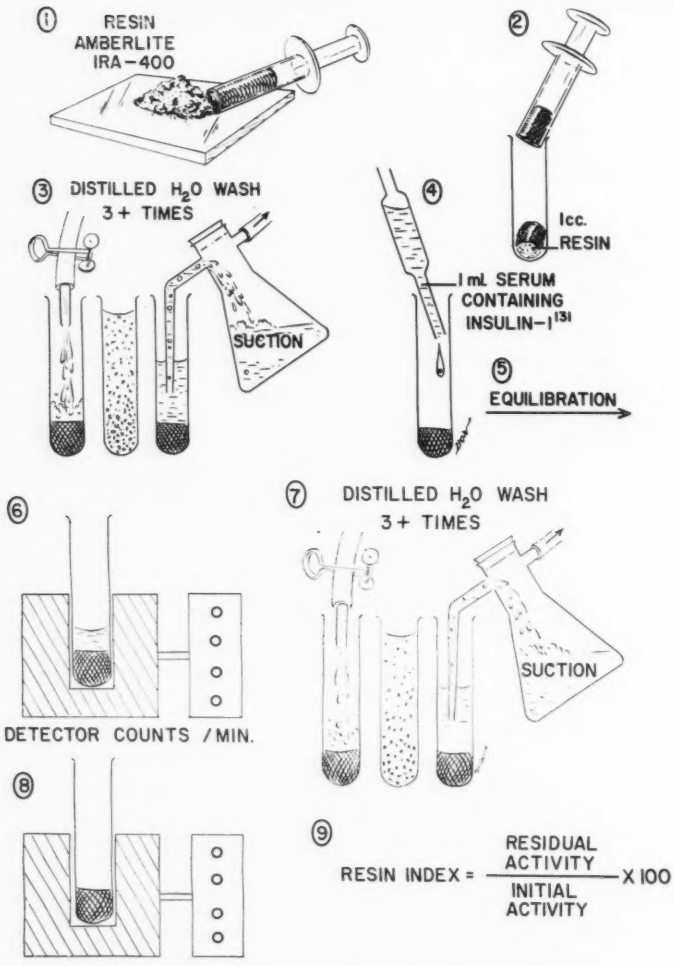
① RESIN AMBERLITE IRA-400
② 1cc. RESIN
③ DISTILLED H_2O WASH 3+ TIMES
SUCTION
④ 1 mL SERUM CONTAINING INSULIN-I^{131}
⑤ EQUILIBRATION
⑥ DETECTOR COUNTS / MIN.
⑦ DISTILLED H_2O WASH 3+ TIMES
SUCTION
⑧
⑨ RESIN INDEX = RESIDUAL ACTIVITY / INITIAL ACTIVITY X 100

Diagram 1. Resin Procedure.

Numbers refer to steps in the manipulation of the resin and the procedure to obtain the resin index.

Following equilibration, the tubes were removed[5] and the radioactivity was measured in a well-type scintillation detector. The resins were then washed three to four times with distilled water in order to remove the radioactivity that was not resin-bound and then recounted. The radioactivity remaining

[5] Occasionally resin particles adhered to the sides of the tubes. These could be easily displaced to the bottom with a stream of water from a wash bottle.

on the resin divided by the initial radioactivity measured the fraction of insulin-I^{131} bound by the resin. This value multiplied by 100 is referred to as the "resin index."

Sera were collected from 53 normal volunteers, 18 patients suffering from diseases unrelated to diabetes, 12 diabetics who had never received insulin, and 50 insulin treated diabetic subjects. The diabetic groups were composed of individuals selected from an out-patient as well as an in-patient hospital population and consisted of adults from both sexes. The daily doses of insulin varied from 7 to 180 units, while the total duration of therapy varied from a few weeks to years. Insulin resistant or juvenile diabetics were not included in this study. In some of the normal subjects, sera were collected pre- and post-prandially, and in the diabetic group, before and after the administration of insulin. Resin indices were also determined on sera which had been enriched with graded quantities of stable insulin[6] before the addition of the labeled insulin. In several experiments, normal saline, 4 per cent human serum albumin, and phosphate buffer (pH 7.3) were substituted for serum.

Bond between resin and insulin

To be sure that the radioactive material bound by the resin was a reliable and reproducible index, it was necessary to ascertain whether the resin values would change with repeated washings. Following equilibration with insulin-I^{131} the resins were subjected to a consecutive series of washes with distilled water and the resin indices were calculated after each single washing. It was apparent from the values obtained (Fig. 1) that the insulin-I^{131} not bound by the resin was removed with the first three to four washings and that further washing had no effect upon the radioactivity remaining on the resin.

Group determinations

The values obtained from the actual determinations performed on the sera from individuals in the several groups are shown in Figure 2. The mean difference between the duplicate resin indices for all the determinations was 1.2 ± 1.0 (standard deviation). The mean resin index of the normal controls (27.0 ± 2.6), and the non-diabetic sick patients (23.0 ± 3.6) is significantly higher than that of the diabetics treated with insulin for more than 12 weeks (11.0 ± 5.6, $P < 0.001$ in each comparison) (5). Some diabetic subjects who had been treated with insulin for less than 12 weeks appeared to exhibit binding of insulin by serum, but the resin indices were not uniformly low (21.0 ± 5.4). Of the group that had received insulin for more than 12 weeks, only three (6%) had resin values in the normal range.

[6] We are grateful to Dr. William R. Kirtley of Eli Lilly Co. for a generous supply of zinc-insulin crystals Lot No. 693502 which assayed at 25.8 units per mg.

TABLE 1. THE EFFECT OF DIET AND INSULIN ON THE RESIN INDICES

Normal subjects resin index			Insulin treated diabetics resin index		
	Fasting	1 hour post prandial		4 hours after insulin	24 hours after insulin
M.M.	25.3	25.5	C.M. 40U NPH	21.7	20.3
M.O.	25.0	26.8	O.B. 15U NPH	9.1	9.2
W.W.	26.3	24.4	C.W. 65U NPH	9.6	9.1

The effect of insulin and diet

There was no appreciable difference in the resin index after meals in normals, and in the diabetics, 4 and 24 hours following the administration of insulin (Table 1).

Influence of the medium

Although the resin was unable to remove more than a fraction of the labeled hormone from serum, it was capable of adsorbing most of the insulin from other media. For purposes of comparison, the resin indices for normal saline, phosphate buffer, normal serum, and 4% human serum albumin are displayed in Figure 3. It is clear that neither saline nor buffer

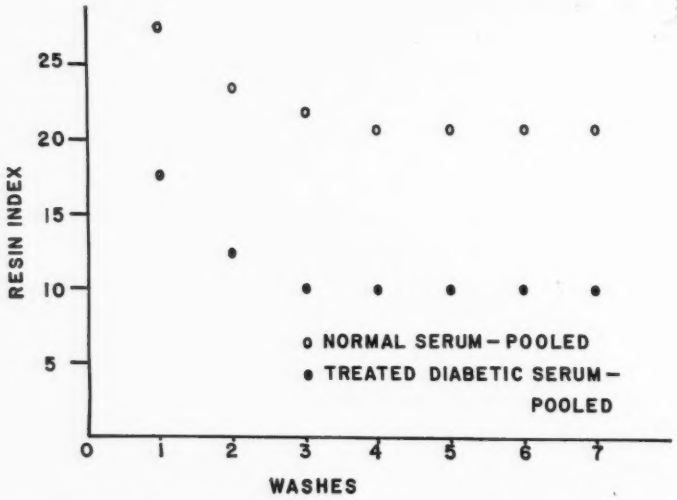

FIG. 1. Resin index as influenced by repeated washing of the resin radioactivity. The resin indices (vertical axis) decline with the first three to four washes (horizontal axis) and then remain constant.

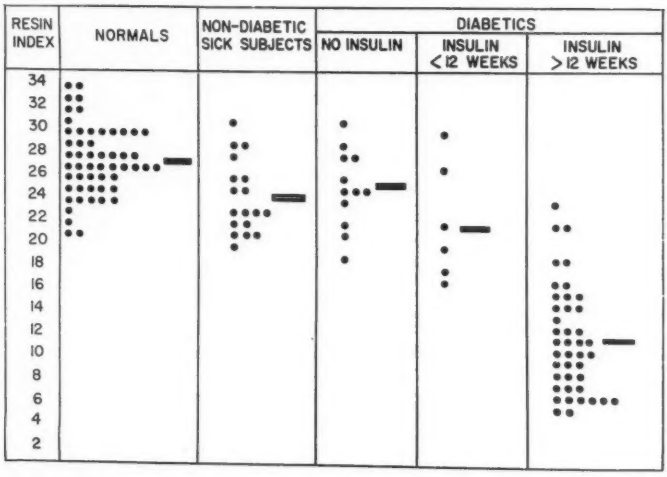

FIG. 2. Resin indices for different groups of subjects.

The closed circle indicates the resin index for a single subject. The solid bar is the mean index for the group.

imposed any major limitations upon the binding capacity of the resin, whereas serum and albumin permitted no more than a minor portion of the insulin to be bound by the resin.

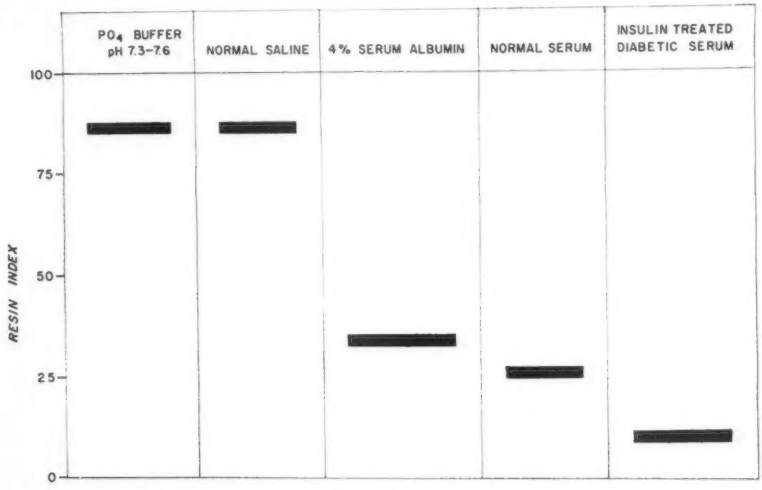

FIG. 3. Variation in resin indices with different media.

The resin index for buffer and saline reflects almost complete binding of the insulin-I^{131} by the resin. Normal serum and serum albumin both result in indices of much lower values.

Resin capacity

After it had been established that the insulin-I^{131} was almost completely bound by the resin in a protein-free solution, studies were carried out to determine the capacity of the resin for stable insulin under similar conditions. Graded amounts of stable insulin (dissolved in HCl, pH 2.7), were added to normal saline before the addition of the labeled insulin. The resin indices were calculated and plotted against the concentrations of stable insulin. From the results, illustrated in Figure 4, it would appear that the maximal binding capacity of the resin is exceeded by more than 1.0 unit of stable insulin, as indicated by the decrease in the indices. These studies were extended to observe resin indices when normal and treated diabetic sera were similarly enriched with stable insulin. Normal serum, under these conditions, displayed no change in resin values until the concentration of more than 1.0 unit of stable insulin per ml. was exceeded. At that point the

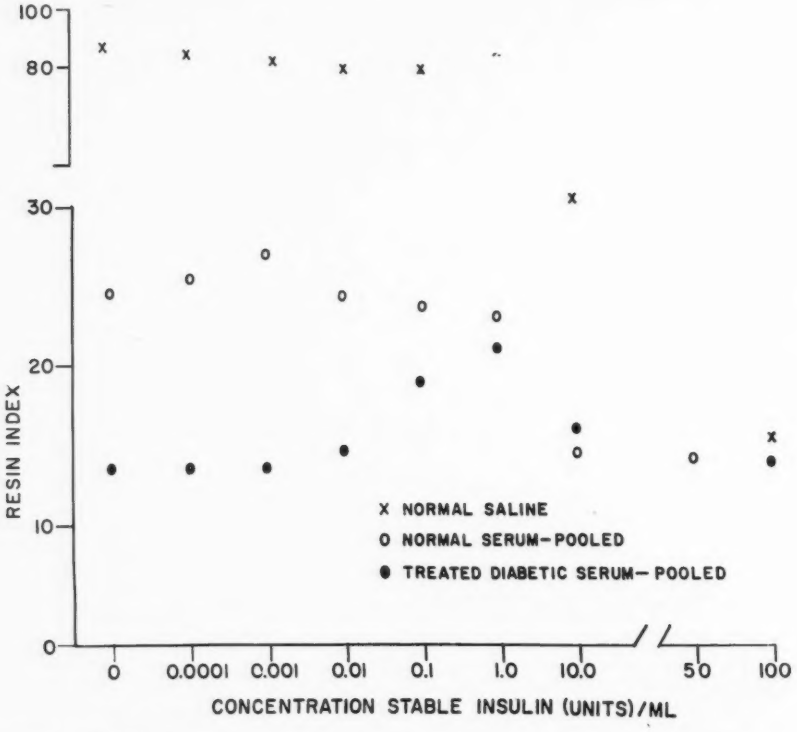

FIG. 4. Enrichment of saline, normal and diabetic serum with stable insulin.

Differences between resin indices (vertical axis) for saline (crosses), normal (open circles), and insulin treated diabetic serum (closed circles) with varying concentrations of stable insulin (horizontal axis).

serum resembled saline in behavior and showed a perceptible decline in the resin indices. Serum from insulin treated diabetics, on the other hand, responded quite differently to the enrichment with stable insulin. The resin indices were enhanced with insulin levels between 0.01 and 1.0 unit, but just as with saline and normal serum, the values dropped with increasingly higher concentrations of stable insulin.

Resin pre-equilibration

The striking differences exhibited between the indices of the protein-free media and the protein-containing media suggested that there was either some degree of insulin-binding by certain proteins of serum, or that the proteins affected the adsorption efficiency of the resin for insulin. In order to define the underlying mechanism involved, one batch of resin was pre-equilibrated with normal serum, another batch with treated diabetic serum, and still a third with 4% albumin. Following this, the resins were washed repeatedly with distilled water, and then re-equilibrated with normal saline containing insulin-I^{131}. The resin indices were determined for each batch of resin and as shown in Figure 5, the values indicate that both the sera and the albumin are capable of modifying the behavior of the resin, effecting diminished insulin binding. The resin index for saline with resin that had not been pre-equilibrated with serum was between 80 and

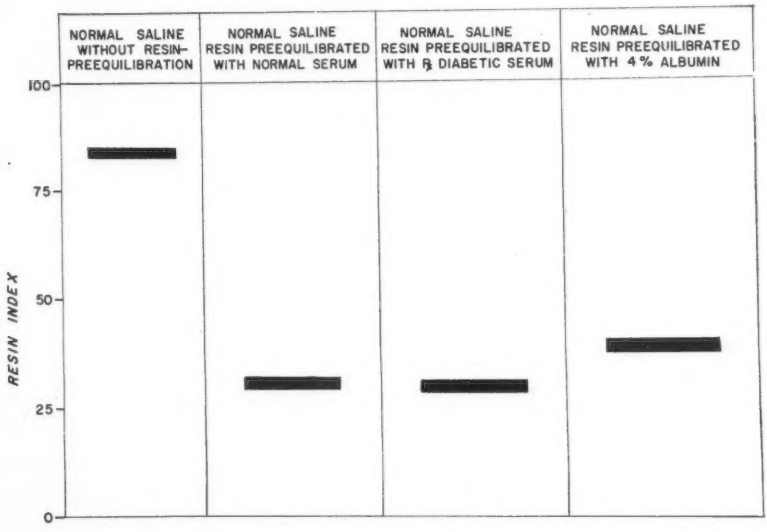

FIG. 5. Resin pre-equilibration with normal serum, insulin treated diabetic serum and 4% human serum albumin.

Resins have been pre-equilibrated with the substances indicated by the column headings before final equilibration with saline containing insulin-I^{131}.

90. The resin index for the saline with the serum-pre-equilibrated resin was the same as the values obtained for normal serum with non-pre-equilibrated resin. This latter observation suggests that the serum possesses either little or no affinity for insulin, otherwise a lower resin index would have obtained for the non-pre-equilibrated resin in the normal serum. These results imply that it is not the competitive binding of the insulin by normal serum that results in the lowered resin index; more likely, the saturation of the resin binding sites with anionic residues from protein fractions preclude making available any unoccupied sites for the insulin molecules.

Resin index versus insulin tolerance

Although the resin indices are the same for normal saline whether the resin is pre-equilibrated with normal or treated diabetic serum (Fig. 5), there is an obvious difference between the resin indices for normal and treated diabetic serum with non-pre-equilibrated resin (Fig. 3). These observations denote that some component (or components) of serum from insulin treated diabetics not only competes with the insulin for the resin binding sites, but in addition competes with the resin for the insulin molecules. Therefore, it seems reasonable to conclude that the serum from insulin treated diabetic subjects contains an insulin binding fraction or fractions. Since the resin index is a reflection of insulin binding by serum from the treated diabetics, the plot of the resin index against the daily dose of insulin administered should define any interdependence or relationship between insulin binding and insulin need. Since insulin requirement may undergo wide fluctuations, the dose of insulin plotted in Figure 6 was the maximal insulin dose required for a period of three consecutive months in the year preceding these studies. Within this framework there was complete absence of correlation between the degree of insulin binding and the insulin requirement in responsive diabetics (correlation coefficient -0.07) (5).

DISCUSSION

The method of interaction analysis by differential adsorption has received scant attention, although this principle was applied over twenty years ago to characterize qualitative differences between the plasma from healthy subjects and patients with nephrosis (6). More recently, a procedure that permitted the quantitation of hormone interaction with serum obtained from a variety of conditions has been described (7). In essence, the method of differential adsorption may be visualized as a system in which the serum proteins are competing for molecules against a synthetic material that has a fixed affinity for the molecules in question. Enhanced binding by the serum proteins will result in diminished resin adsorption and conversely, weak protein binding will encourage maximal resin binding.

If this assumption is correct, then in the case of "free insulin" at pH above 5.3, where it possesses a net negative charge, the appropriate basic resin should atract the major share of the hormone that is not bound by serum protein. The validity of this proposition appears to be substantiated by a resin index between 80 and 90 when labeled insulin is permitted to equilibrate with the Amberlite resin in a protein-free solution.

Binding by normal serum

Although "insulin-like" activity has been identified with the alpha$_1$ globulin and albumin fractions (8), conventional techniques have failed to show any preferential association of insulin-I^{131} with a specific protein frac-

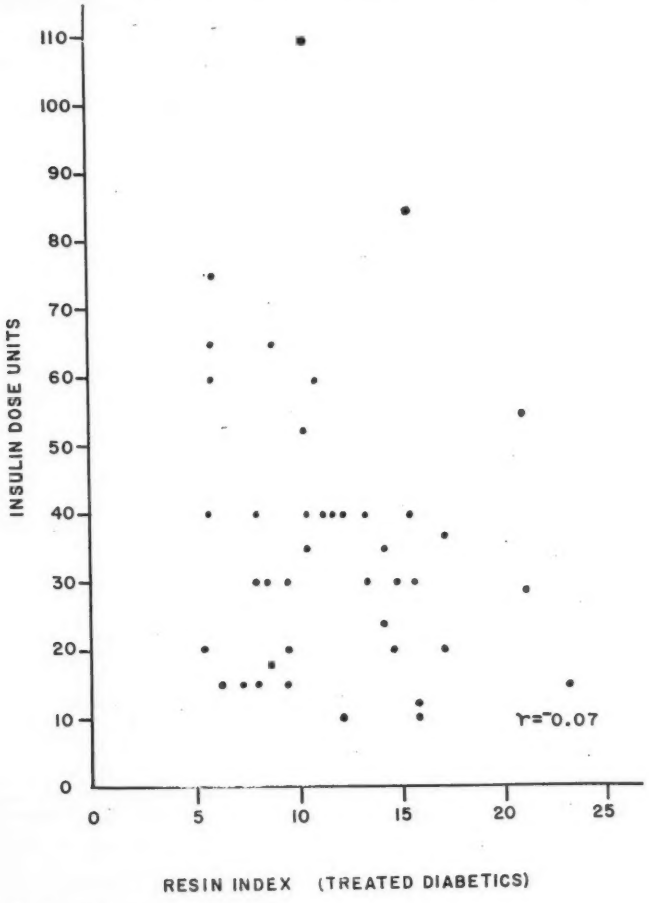

FIG. 6. Resin index versus insulin dose.

The resin index (horizontal axis) for insulin responsive diabetics is plotted against the daily insulin dose (vertical axis).

tion in normal serum (1, 3). The inability to relate insulin-I^{131} to a selective serum protein perhaps may be ascribed to a weak linkage between the existing complex which becomes fractured upon technical manipulation. Whether insulin circulates in the free state or in loose combination with a protein or proteins would not appear to conflict with results alluded to earlier, which inferred that normal serum does not reduce the resin index by virtue of competitive binding of insulin. It seems more reasonable to conclude that the depressed value is a manifestation of a limited resin capacity for insulin-I^{131}, resulting from the saturation of the resin lattices with anionic residues of some component of serum. This process would serve then to exclude the insulin molecules from sites which would otherwise be unoccupied by the anionic residues. The possibility that the components which fill the resin pores are themselves active binders of insulin is not entirely excluded but appears to be unlikely as shown by the failure of the resin index to decrease when less than 1.0 unit of stable insulin was added to normal serum (Figure 4).

Binding of serum from diabetics

Any explanation for the significantly lower resin values of sera from insulin treated subjects must invoke still another consideration. The affinity of the insulin molecule for any particular anionic resin is determined, to some extent, by the number of net negative charges being carried. This in turn is dependent upon the isoelectric point of insulin and the pH of the solution. The less alkaline the pH with respect to the isoelectric point of insulin, the fewer the net negative charges and the weaker becomes the attraction to the basic resin. It has previously been shown that sera from insulin treated diabetics contain antibodies to insulin (1). Thus, the insulin-antibody complex might possess an isoelectric point much closer to the pH of the serum. This would result in a suppression of the negative charges and the consequent loss of affinity between resin and complex. This hypothesis is strengthened by the observation that *cationic* resins are unable to bind "free insulin" (added to saline) but are capable of removing "insulin-like" activity from plasma (8). This suggests one or more of the following possibilities: 1) the insulin is bound to a protein or to some constituent of plasma which is removed by the resin, 2) endogenous insulin circulates in a state different from that of exogenous insulin, 3) I^{131} labeled insulin and unlabeled insulin may behave differently.

Insulin enrichment

The addition of small amounts of unlabeled insulin (less that 1.0 unit per ml.) to either saline or normal serum did not appear to affect the resin indices, whereas larger concentrations resulted in a decrease in the values. However, when serum from treated diabetics was enriched with 0.01 to 1.0 unit of stable insulin per ml. there was a decided elevation in the resin

index; but just as with the saline and normal serum, higher concentrations of insulin produced diminished indices. These observations suggest that 1) the insulin binding capacity of the resin in saline, normal serum, or diabetic serum does not exceed 1.0 unit of stable insulin per ml. and 2) sera from insulin treated diabetic subjects contain insulin binding protein (antibody?) that can effectively exchange the labeled insulin for the stable, thereby permitting the former to be bound by the resin. The diminished resin indices produced by the addition of larger concentrations of unlabeled insulin, in all likelihood, result from the marked reduction in specific activity. It is of interest that exchange of the resin-bound labeled insulin for the unlabeled insulin does not occur as shown by the failure to remove the insulin-I^{131} by treatment with large concentrations of stable insulin, although labeled and unlabeled insulin appear to be freely exchangeable in normal serum (9).

The lack of correlation between insulin binding and insulin requirement in diabetics not resistant to insulin is clearly evident in these studies. This observation is in agreement with similar findings made by others using different techniques (3, 4), although some reports have described still greater degrees of binding in sera from insulin resistant diabetics (10, 11). Since the studies reported here do not include resistant subjects, further comments regarding possible binding differences must be held in abeyance until the sera from grossly insulin resistant diabetics are examined. Thus, within the limitations of our studies it would appear that the insulin binding protein in the responsive patients does not participate in the determination of the insulin requirement.

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NOTES AND COMMENTS

THE GROWTH HORMONE CONTENT OF SEVERAL VERTEBRATE PITUITARIES[1]

ABSTRACT

The growth hormone potencies of pituitaries obtained from fish, frog, turtle, chicken, rabbit, cat, horse, and whale were compared in the tibia test assay. A significant response was obtained with frog, horse, rabbit, whale, and cat pituitary suspensions. The pituitaries of chickens and turtles caused a small but nonsignificant increase in epiphyseal width, whereas fish pituitaries had no growth activity in the hypophysectomized rat.

Recent physiological and biochemical studies on various growth hormone preparations have led to the concept that growth hormone is at least partially species specific (1–6). Investigations of the chemical properties of growth hormone preparations from ox, sheep, horse, pig and fish pituitaries (6) have shown that the isoelectric points and sedimentation constants differ; and study of beef, human and monkey pituitary extracts (5) revealed differences in molecular weight, amino acid composition, and isoelectric point of the growth hormones analyzed. Reports on the effect of administration of pituitary growth hormone preparations from one species on the growth response of another species demonstrated that hypophysectomized tadpoles responded to beef growth hormone (8), and that transplants of rabbit pituitaries caused growth in hypophysectomized puppies (9). Beef growth hormone preparations are ineffective in normal and hypophysectomized guinea pigs (10), but beef pituitaries are more effective than fish pituitaries in increasing the body weight and length of hypophysectomized killifish (11). In primates, beef and hog growth hormone extracts do not alter the growth of normal and hypophysectomized monkeys (2), whereas the hypophysectomized monkey (3) and hypopituitary dwarf (12) respond to growth hormone preparations of primate origin. In hypophysectomized rats, purified fish growth hormone was without effect in the tibia test (6), but a positive growth response was obtained with amphibian pituitary extracts (1); and the growth hormone potency of the whale pituitary was reported to be similar to that of cattle hypophyses (13, 17). Human pituitary (14) and plasma (15) growth hormone preparations, and monkey pituitary extracts (16), elicit a positive response in the tibia test.

These observations on the relative specificity of the growth hormones suggested the following inquiry into the response of the hypophysectomized rat to the growth hormone activity of several vertebrate pituitaries.

MATERIALS AND METHODS

The whole pituitaries were removed from shad, frogs, turtles, chickens, horses, cats, and rabbits as soon as possible after the death of the animals and placed in absolute acetone, dried, and stored in a vacuum desiccator for use in the tibia test assay for growth hormone. Whale pituitary material was received as an acetone-dried powder of the anterior lobe.[2] The growth hormone potency of the pituitary material was determined according to the method of Evans *et al.* (7). For each assay, three groups of hypophysectomized female rats were injected with graded doses of a purified beef growth hormone preparation (Armour and

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[2] The authors are indebted to Dr. Knut Tjønneland of Nyegaard & Co. A/S, Oslo, Norway for the whale anterior pituitary powder.

TABLE 1.* GROWTH HORMONE ASSAYS OF VERTEBRATE PITUITARIES

Donor of Pituitary Preparation	Dose Pituitary Powder	Recipients	Epiphyseal Width	
	μg./day	No. of hypox. rats	Micra Mean ± S.E.	
Chicken	2000	4	163 ± 10.4	
Beef growth hormone	10	3	154 ± 12.0	s = 20.76
Beef growth hormone	25	5	192 ± 9.3	b = 96.58
Beef growth hormone	50	5	221 ± 9.3	λ = 0.152
Untreated hypox. rats	—	4	146 ± 10.4	
Rabbit	2000	5	232 ± 10.8	
Cat	2000	5	198 ± 10.8	s = 24.14
Beef growth hormone	10	5	179 ± 10.8	b = 85.92
Beef growth hormone	25	6	195 ± 9.9	λ = 0.220
Beef growth hormone	50	6	240 ± 9.9	
Untreated hypox. rats	—	5	142 ± 10.8	
Frog	2000	5	217 ± 12.9	
Shad	2000	6	164 ± 11.8	
Horse	2000	5	254 ± 12.9	
Turtle	2000	5	180 ± 12.9	s = 28.82
Whale	2000	6	206 ± 11.8	b = 114.80
Beef growth hormone	10	6	230 ± 11.8	λ = 0.252
Beef growth hormone	25	6	268 ± 11.8	
Beef growth hormone	50	5	312 ± 12.9	
Untreated hypox. rats	—	6	157 ± 11.8	

s = standard deviation for the assay.
b = slope of standard.
λ = s/b.
S.E. = standard error of the mean ($s\sqrt{1/n}$).

* The authors would like to acknowledge the kind assistance of Dr. Mindel C. Sheps for her advice on the statistical methods.

Co., No. 732–181); the dried donor pituitaries were weighed, homogenized with saline, and injected subcutaneously at a concentration of 2000 μg (dry weight) per cc. per day for four days. At necropsy, the right tibia was removed from those rats in which gross inspection of the sella turcica and organ weights evidenced complete hypophysectomy.

RESULTS

The epiphyseal widths and statistical evaluation of the response of hypophysectomized rats to the various pituitary preparations are shown in Table 1. Crude extracts of whole pituitaries obtained from frog, rabbit, cat and horse, and an anterior lobe preparation of whale pituitary had significant growth hormone potencies. Turtle and chicken pituitary extracts induced a measurable but nonsignificant growth stimulation, whereas shad pituitary material failed to elicit a response.

COMMENT

The determination of growth hormone potencies of several vertebrate pituitaries was limited, in these experiments, to the rat tibia test. The cartilagenous plate of the hypophysectomized rat was clearly more sensitive to amphibian and mammalian growth hormones than to fish, reptile and bird hormones. The delineation of the species relatedness of the growth hormones will require much additional study.

JOLANE SOLOMON AND ROY O. GREEP

Biological Research Laboratories
Harvard School of Dental Medicine
Boston, Massachusetts

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IN VITRO METABOLISM OF D-THYROXINE[1]

ABSTRACT

Comparisons were made of the metabolism of D- and L-thyroxine by enzyme systems prepared from rat kidney. With slices, D-thyroxine, unlike L-thyroxine, was not deiodinated to triiodothyronine. With the mitochondrial enzyme system D-thyroxine was unaltered while L-thyroxine was actively converted to tetraiodothyroacetic acid. It is possible that the failure of D-thyroxine to undergo these transformations may account for its metabolic inactivity.

Interest has been raised recently in the pharmacological effects of D-thyroxine. This stereoisomer of the natural hormone, L-thyroxine, has been reported to suppress the TSH production by the pituitary (1) and to lower the serum cholesterol levels (2). Doses used were very high (1–20 mg.) when compared with the usual doses of L-thyroxine (0.1–0.3 mg.), and yet were without noticeable effect in raising the metabolic rate. This is in accord with previous reports which indicate that D-thyroxine has little, if any, effect on tissue metabolism (3).

It has been proposed that thyroxine undergoes chemical transformation *in situ* before becoming active at the cellular level. The deiodination of L-thyroxine to L-triiodothyronine and the conversion of these compounds to tetraiodothyroacetic acid (tetrac) and triiodothyroacetic acid (triac) respectively are reactions observable *in vitro* which may be involved (4, 5).

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Since tetrac and triac, or any of the predicted intermediates in their formation (i.e., the keto-acid and aldehyde) do not have optical activity and could theoretically be derived from either the D- or L- form of thyroxine, it is of interest to compare reactions of these stereoisomers in *in vitro* systems known to metabolize L-thyroxine. The physiologic significance of these reactions would be questionable if a metabolically inactive isomer, D-thyroxine, were found to undergo these transformations.

Slice Experiments

Since L-thyroxine is deiodinated to L-triiodothyronine by rat kidney slices this tissue was chosen for the present experiment. Slices were prepared from the kidneys of normal 200 gram male rats. They were incubated for three hours at 37 degrees C. in Krebs-Ringer phosphate solution to which 0.01 micrograms of I^{131} labelled L-thyroxine or D-thyroxine was added. Slices which had been boiled for five minutes were used as controls. At the completion of incubation the slices were removed, rinsed, and homogenized in 2 ml. of distilled water. The homogenate was extracted with 10 volumes of 3% NH_4OH in butanol. The extract was concentrated to 0.5 ml., triiodothyronine was added as a marker, and the sample was dried on Whatman #3 MM paper for chromatography. The solvent system used for chromatographic separation was T-amyl alchohol saturated with 2N NH_4OH. With this solvent system the Rf values of the compounds of interest are: thyroxine 0.20, tetraiodothyroacetic acid 0.30, triiodothyronine 0.40 and triiodothyroacetic acid 0.56. Iodide has an Rf of 0.16. After the solvents had run in descent for 24 hours the paper was dried in air. The radioactive components were located on the chromatogram with a Geiger counter equipped with a continuous automatic recording scanner. The position of marker triiodothyronine was located by color development with 4-amino-antipyrine.

The results are shown in Figure 1. As in previous experiments, appearance of the label at the site of the triiodothyronine marker indicated deiodination of L-thyroxine to L-triiodothyronine. Iodide liberated by the deiodinating system remains with the residue during the extraction procedure accounting for the absence of an iodide peak on the chromatogram. In the D-thyroxine experiment no radioactivity appeared at the position of the triiodothyronine marker. Therefore, it appears that D-thyroxine, unlike L-thyroxine, is not deiodinated to triiodothyronine under these conditions. Whether this reflects an absolute stereoisomeric specificity of enzyme for substrate, or only a relatively greater affinity of the enzyme for endogenous L-thyroxine present in the slices cannot be determined.

Mitochondria Experiments

An enzyme system has been prepared from the mitochondria of rat kidney which regularly converts L-thyroxine to tetraiodothyroacetic acid and L-triiodothyronine to triiodothyroacetic acid (5). Therefore, the kidneys of normal male rats, homogenized in sucrose, were used as a source of enzyme. The mitochondria were isolated by the procedure of Schneider (6), suspended in 0.25 M sucrose and disrupted in a Raytheon 10 kc. sonic oscillator for 5 minutes with alcohol at −5 degrees C. as a coolant. The debris and undisrupted mitochondria were removed by centrifugation at 100,000 G. for 60 minutes. The enzyme system, remaining in the supernatant, was dialyzed for 3 hours against cold distilled water. To each reaction flask was added: 5 ml. phosphate buffer (0.1 M, pH 7.4), 30 micromoles DPN, 20 ml. of enzyme solution (80 mg. of protein) and 0.1 microgram I^{131} labelled L-thyroxine. One and one half milligrams thyroxine (not labelled), sufficient to permit identification of reaction products by color development on chromatograms, was used as a substrate. L-thyroxine was added to one flask, D-thyroxine to the other. The flasks were incubated at 37 degrees C. for 2 hours. Following

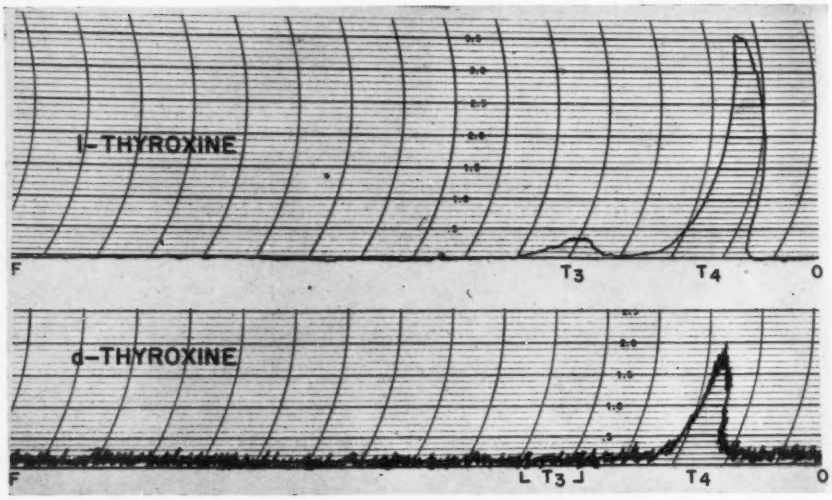

FIG. 1. Slice experiment comparing deiodination of L-thyroxine and D-thyroxine to triiodothyronine.
O—origin of chromatogram
T_4—thyroxine radioactivity
T_3—triiodothyronine marker
F—chromatographic front

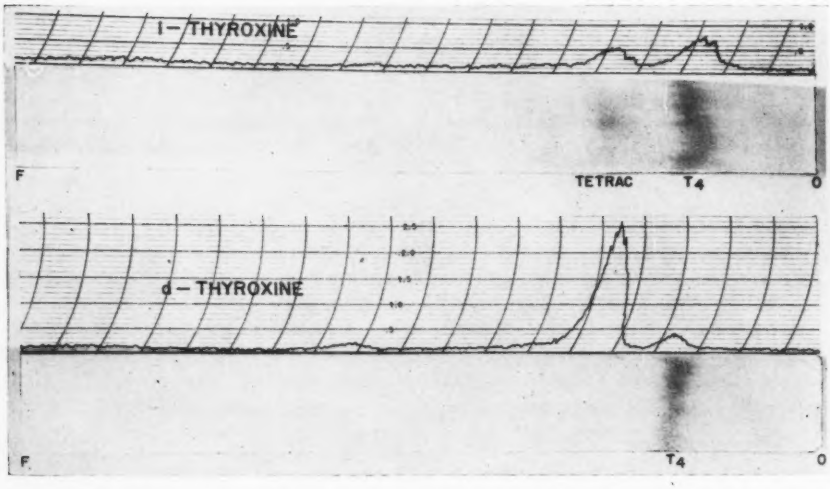

FIG. 2. Mitochondria experiment comparing reactions of L-thyroxine and D-thyroxine.
O—origin
T_4—position of thyroxine
Tetrac—position of tetraiiodothyroacetic acid
F—chromatographic front

incubation the reaction mixtures were extracted with 10 volumes of 3% NH_4OH in N butanol, the volume reduced to 0.5 ml. and dried on Whatman #3 MM paper. No marker compounds were added. Chromatographic procedure, radioactivity analysis and color development were carried out exactly as described for the slice experiments.

Results are seen in Figure 2. The radioactivity data are derived from the tracer quantity of I^{131} labelled L-thyroxine which served as an internal control. The appearance of radioactive tetrac proves that the enzyme system was active in each flask. In the D-thyroxine experiment the conversion of tracer L-thyroxine to tetrac was almost complete. In the L-thyroxine experiment less labelled tetrac appeared due presumably to competition for the enzyme by unlabelled L-thyroxine.

The chromatographic data are derived from the unlabelled substrates. In the D-thyroxine experiment only one color band appeared. This had the chromatographic mobility of thyroxine and represents unchanged substrate. In the L-thyroxine experiment in addition to the thyroxine band a second color band appeared which had the chromatographic characteristics of tetrac.

D-thyroxine is not deiodinated to triiodothyronine by rat kidney slices nor converted to tetraiodothyroacetic acid by rat kidney mitochondria. Both of these reactions can be readily demonstrated with L-thyroxine. It is possible that these observations account for the metabolic inactivity of D-thyroxine.

FRANK C. LARSON, KENKICHI TOMITA AND EDWIN C. ALBRIGHT
Department of Medicine and Institute for Enzyme Research
University of Wisconsin
Madison, Wisconsin

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DEGENERATION OF OVA IN THE RAT AND RABBIT FOLLOWING ORAL ADMINISTRATION OF 1-(P-2-DIETHYLAMINOETHOXYPHENYL)-1-PHENYL-2-P-ANISYLETHENOL[1]

ABSTRACT

A non-steroidal compound, MER-25, 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-anisylethanol, was administered orally to mated female rats during the period of tubal passage of ova. In animals sacrificed and examined on day 3 after mating, the number of ova found in rats that had received higher doses was very small, but the number of degenerated ova found in the tubes was not significantly high, suggesting that degeneration of tubal ova may be very rapid. In animals

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examined on days 14–16 after mating, there was more degeneration before implantation than had been indicated by the appearance of 3 day ova, showing that physiological disturbances occurred earlier than morphological degeneration. Complete degeneration was observed in rats that had been treated only once, on day 2 or day 3, at 30–60 mg./kg. Rabbits were fed with MER-25 daily for 4 days at 50 mg./kg., or at 100 mg./kg. on day 0, or 25 mg./kg. on day 1. Forty-one to 59 percent degeneration of ova was observed in these cases.

It has been reported by Segal and Nelson (4) that MER-25, 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-anisylethanol, induces rapid degeneration of ova in the tubes of rats fed with this non-steroidal compound on the day after mating and throughout the normal period of tubal passage of ova. A compound with such a specific effect is of great interest for the understanding of animal reproduction and in the application of antifertility measures. This note reports our observations of the effect of MER-25 on: (a) the tubal ova of rats fed with various doses after mating, (b) the embryos of rats given varying amounts on different days after mating, i.e. day 1, 2, or 3, and (c) on the blastocysts and embryos of rabbits fed with various doses at the time of tubal passage of ova.

ANIMALS AND METHODS

Female rats of the Sprague-Dawley strain, at body weight of about 200 gm., were caged with fertile males overnight. Vaginal smears were examined for sperm the following day. When sperms were found in the smear, it was considered day 1. MER-25 was force-fed on a body weight basis through a catheter connected to a syringe. The compound was either dissolved in 0.1–0.47N HCl (according to concentration and in most cases) or suspended in propylene glycol. In the study of the tubal ova, rats were treated in the mornings of day 1 through day 3 and sacrificed in the evening of day 3. One Fallopian tube was flushed with saline by means of a capillary pipette connected to an air pressure line (1). The other tube was fixed in Bouin's solution, sectioned in complete series, and stained with hemotoxylin and eosin. To study events after tubal passage of ova, rats were treated only once, in the morning of day 1, 2 or 3, and sacrificed 14–16 days after mating. Counts made of degenerations after implantation and of normal embryos gave the total number of implantation sites. By comparing this with the total count of corpora lutea, the number of degenerations before implantation was deduced.

In studying blastocyst degeneration in MER-25 treated rabbits, females of mixed breeds, but mainly of Dutch stock, were artificially inseminated with undiluted semen of fertile males and given an injection of pituitary extract to induce ovulation. Soon afterward, they were fed with MER-25 by means of a glass tube inserted into the back of the mouth. The day of insemination was considered day 0, the day after insemination considered day 1 of pregnancy. The animals were sacrificed 4 to 14 days after insemination, and the uteri flushed with saline for the recovery of blastocysts, or examined for the numbers of degenerating and normal embryos.

RESULTS AND DISCUSSION

Degeneration of tubal ova in the rat. Ten rats were fed with MER-25 at 50 mg./kg. three times, on days 1, 2 and 3, or at 100 mg./kg. or at 200 mg./kg. once on day 1. The number of ova recovered from tubes or found in the histological section of these 10 rats was very small, an average of 1.6 ova per rat. Of these 16 ova, only two were degenerated (12.5%). In 7 rats fed at 25 mg./kg. or at 50 mg./kg. etc. twice, on day 1 and 2, an average of 6 ova per rat were found in the evening of day 3, and of these 41 ova 3 (7.3%) were degenerated. The tubes of 5 control rats contained an average of 8 ova per rat, and of these 41 ova, 3 were degenerated (7.3%). From these results it seems that although the proportion of morphologically degenerated ova is not significantly high the disappearance of ova in rats fed with a higher dose indicates rapid degeneration of tubal ova.

Degeneration of embryos in the rat. Table 1 presents the results when rats received MER-25 only once and were examined 14–16 days after mating. These data show the following points: (a) The higher the dose-level, the more effective is the induction of embryonic degeneration. (b) Feeding once on day 2 or day 3 is more effective than feeding on day 1. (c) Most ova degenerated before implantation, indicating a harmful effect

TABLE 1. EFFECTS OF MER-25 ON THE EMBRYONIC DEGENERATION IN THE RAT

Dosage	Feeding on	No. of rats	Total no. of C. L.	No. of deg. before impl.	No. of deg. after impl.	No. of normal embryos
15 mg./kg.	day 1	10	119	50 (42%)	11 (9.2%)	58 (48.7%)
	day 2	8	95	85 (89.5%)	4 (4.2%)	6 (6.3%)
	day 3	6	73	48 (65.8%)	13 (17.8%)	12 (16.4%)
30 mg./kg.	day 1	6	72	26 (36.1%)	9 (12.5%)	37 (51.4%)
	day 2	6	71	63 (88.7%)	4 (5.6%)	4 (5.6%)
	day 3	6	69	49 (71.1%)	20 (28.9%)	0 (0%)
60 mg./kg.	day 1	6	69	64 (92.8%)	3 (4.3%)	2 (2.9%)
	day 2	6	74	74 (100%)	0 (0%)	0 (0%)
	day 3	6	71	58 (81.7%)	13 (18.3%)	0 (0%)
Control		10	116	3 (2.6%)	2 (1.7%)	111 (95.7%)

on ova during their passage through the tubes. (d) Since the proportion of morphologically degenerated ova is much lower when examined on day 3 (7 to 13%, see above) than that of degenerated embryos when examined on day 14–16 (48–100%), see Table 1, it seems that physiological deterioration of tubal ova occurs before morphological degeneration becomes noticeable.

Degeneration of blastocysts in the rabbit (Table 2). An inconsistent effect was noted when MER-25 was administered at 25 mg./kg. in four daily doses after artificial insemination. There was no degeneration of blastocysts in 4 animals, although all eight blastocysts of another animal degenerated. A single dose of 100 mg./kg. administered on day 1, or 4 daily doses at 50 mg./kg. given on days 0 through 3, caused degeneration of all blastocysts in 5 out of 15 rabbits. Possibly, differences in absorption of this compound account for the inconsistency. Within the limitations of the inconsistent results, however, there is an indication that the compound was more effective when administered at 25 mg./kg. once on day 1 than when a single dose of the same amount was given on day 3. The toxicity of the compound is revealed by the fact that 3 out of 23 rabbits died following the feeding of MER-25 at 25–50 mg./kg. for four days and after receiving 100 mg./kg. for 1 day. However, at 25 mg/kg. for 1 day all 10 animals survived.

Other observations. When rabbit ova at the pronuclear stage were cultured in rabbit serum containing 0.025 to 0.1% of MER-25, no segmentation was observed. It is possible, therefore, that the incomplete effectiveness of the compound *in vivo* may be due to inadequate concentration in the Fallopian tubes. Rabbit sperm suspended in saline containing 1 to 2% MER-25 showed greatly depressed motility but no loss of fertilizing capacity. Ova fertilized by sperm so treated developed normally. Moreover, there was no effect

TABLE 2. EFFECTS OF MER-25 ON EMBRYONIC DEGENERATION IN THE RABBIT

Dosage	No. of female rabbits	Examination, days after insemination	Total No. of C.L.	Total No. of blastocysts or embryos	Total No. of degeneration	% degeneration
25 mg./kg. On day 0, 1, 2 and 3	5	4–10	43	40	8	20
50 mg./kg. On day 0, 1, 2 and 3	8	4–10	70	61	25	41
100 mg./kg. On day 0	7	6–10	58	39	23	59
25 mg./kg. On day 1	5	14	39		18	46
25 mg./kg. On day 3	5	14	55		15	27
Control	5	4–6	55	49	1	2

upon the fertilization or development of ova in rabbits administered with MER-25 orally one day before mating, or vaginally one day after mating. These facts indicate that MER-25 adversely affects only the ova through the system to tubes and that it has no effect on the capacitation of sperm in the Fallopian tubes (2).

Although MER-25 blocks the response of some estrogen target organs to the stimulating action of estrogens (3), it is unlikely that its anti-zygotic activity is due to such an action. We have observed that pronuclear rabbit ova develop into morulae when transferred into the Fallopian tubes of rabbits that have been ovariectomized for as long as 24 days. The findings of Segal and Nelson (4) however are amply confirmed.

M. C. CHANG

Worcester Foundation
Shrewsbury, Massachusetts
and
Department of Biology, Boston University
Boston, Massachusetts

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